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v. 14

CEREAL CHEMISTRY

VOL. XIV JANUARY, 1937 No. 1

SOLUBLE STARCH: A STUDY OF ITS PROPERTIES WITH A RECOMMENDED SUPPLEMENTARY PROCEDURE FOR DETERMINING ITS SUITABILITY FOR USE IN THE LINTNER DETERMINATION

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(Read at the Annual Meeting, June 1936)

Introduction

The determination of diastatic power by the Lintner method in many of its modified forms is a very controversial subject. Aside from the fact that the utmost care and skill must be exercised in conducting the Lintner value determination, there are factors with respect to the completion of the determination which can introduce serious analytical errors. These factors may be broadly subdivided into three groups. In one group such factors as purity of the reagents, purity of the distilled water, cleanliness and solubility of the glassware, alkalinity of the filter paper, absence of acid and other laboratory fumes have been mentioned as significant.

A second group of factors are related to the analytical procedure in the Lintner value determination. Among these factors may be mentioned the time, temperature, and extent of stirring of the malt-infusion; the time and temperature relation of the malt-extract-starch suspension; failure to adhere to Kjeldahl's (1876–82) Law of proportionality; disregard of the deterioration of Fehling's Solution; failure to run blank determinations as well as failure to determine the personal error equation in relation to the particular sugar conversion table used in connection with the specified method.

In the instance where a volumetric procedure for sugar analysis is used factors such as the degree of dilution, the surface area and depth of the boiling solution, the atmospheric pressure and temperature of the

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boiling solution, as well as the back oxidation of cuprous oxide, are important.

A third and highly important set of factors influencing the accuracy of the Lintner value determination is associated with the quality of the soluble starch used in the determination. The purity of the soluble starch, the purity of the prepared soluble starch solution, the pH of the original starch solution, the effect of buffering as well as the importance of the use of fresh solutions of soluble starch are all factors of great importance and should be controlled in order to provide for the greatest precision when making the test.

Experimental

There is little excuse for not overcoming the difficulties listed as the first group of factors, in fact these factors should be the first order of consideration of the careful analyst.

While all of the factors listed in the second group are important, it is believed, and the belief is fortified by experience from extensive trials with the Lintner method, that the most extensive source of error associated with the test is in connection with the soluble starch used. Starches which are over- or under-converted, starches which precipitate on standing, starches of improper pH, have all given trouble in the Lintner value determination in these laboratories.

Studies were, therefore, concentrated on an analytical study of the characteristics of the soluble starch now in general use in connection with Lintner determinations. However, the research was not intended so much to study all of the chemical properties of soluble starch as to study those chemical properties having an influence on the Lintner determination. Such properties as the acid and alkaline condition of the soluble starches, the clarity of the prepared solutions, the susceptibility of the starch to diastase, the ash content of the starch, the quantity and character of the reducing substances present, were all factors considered of significant importance in relation to the Lintner value determination to warrant their individual study.

The Ideal Soluble Starch

The ideal soluble starch is one that gives a clear opalescent solution with no precipitation of amylose on standing or after the addition of a buffer solution. The pH of the unbuffered starch solution should be 4.6 (this value would be the neutral point for starch in the sense that water is neutral at a pH of 7.0). The ideal soluble starch should contain no reducing substances and should be absolutely free from erythrodextrin. The starch should also be absolutely susceptible to diastase. It should be of a low ash content and free from or containing only traces of phosphates.

A starch with such analytical qualifications does not exist in all probability (at least commercially), and it is with the view towards finding to what extent various commercial starches approach the ideal starch that the investigations herein reported were undertaken.

For comparative purpose the chemical characteristics of a number of specially prepared soluble starches were studied in order to determine whether soluble starch of the "ideal sort" can, in effect, be made.

Soluble Starches Used in the Study

Twelve soluble starches were studied in the investigation. Three starches were of commercial grade (according to Lintner); four starches were of commercial indicator grade, while five starches were specially prepared. Identification of the starches will be found in Table I.

TABLE I

IDENTIFICATION OF THE STARCHES USED IN THE INVESTIGATION

Sample number	Nature	Origin	Labeled
1	Commercial	Potato	According to Lintner
2	"	"	" " "
3	"	"	Improved Lintner
4	"	?	Indicator Grade
5	"	?	" "
6	"	Potato	Powder
7	"	?	Practical
8[a]	Specially prepared	Potato	—
9[b]	" "	Corn	—
10[b]	" "	Potato	—
11[c]	" "	Corn	—
12[c]	" "	Potato	—

[a] Prepared according to Small's method (1919).
[b] Prepared according to Small's method by Dr. Allan Dickson of the University of Wisconsin.
[c] Prepared by Dr. Dickson by the nitric acid method.

Titratable Acidity and pH Values of Soluble Starches

Much study has been devoted to the alkalinity and acidity of starch and the desired pH for optimum susceptibility to diastase. Soluble starches are quite easily adjusted to pH 4.5 to 4.7 by means of acetic acid-sodium acetate buffer solutions. Ford's (1904) exhaustive study on acidity and alkalinity of starch gives much information on the effect of the presence of many alkalis, acids, and salts. His outstanding conclusions are (1) that a starch that is alkaline to methyl orange and neutral or acid to rosolic acid will give normal maltose productions; (2) that alkaline phosphates hinder diastatic action; (3) that acid phosphates contribute a negligible amount of acidity to starch solutions; (4) that starches alkaline to rosolic acid retard diastatic activity; (5) that traces of phosphates of the type MH_2PO_3 are only feebly acid

and do not influence diastatic activity whereas a phosphate of the type M_2HPO_3 dissociating into $M_2 + HPO_4$ and $HPO_4 + H_2O = H_2PO_4 + OH$ renders the solution alkaline and creates an unfavorable environment for diastatic action.

Gore (1925) concluded that a pH range from 4.5 to 5.5 was optimum for a mixture of soluble starch and a diastase infusion, Sherman, Thomas and Baldwin (1919) have also presented data which substantiates in a general way those reported by Gore, namely, that optimum diastatic activity takes place when the pH of the soluble starch is between pH 4.4 and 4.5.

The problem with respect to the acidity of soluble starch for the purposes of the Lintner test is not, however, its adjustment to a pH of 4.5 to 4.7 by means of buffers, but rather which starch is more desirable —the one close to the starch neutral point on the acid side, or the one close to the starch neutral point on the alkaline side.

In Small's preparation of soluble starch, for example, $NaHCO_3$ is used in neutralization of the acid (HCl) at the end of the conversion with methyl orange as an indicator. Some methyl orange indicators have a pH range of from 2.9 to 4.0 and others pH 3.1 to 4.4.

Although methyl orange may be used in the presence of CO_2 it is not a good indicator. Its end point is indistinct and as a result a starch solution may be neutral to methyl orange but the starch yet be acid. If the neutralization is carried too far $NaHCO_3$ may be present with a resulting high pH value. Also free $NaHCO_3$ is precipitated with alcohol and the starch preparation is difficult to purify and any excess of $NaHCO_3$ in the starch would inhibit diastatic activity. Illustrative of this is sample No. 8 which was prepared by Small's method. The pH of this sample was 6.3.

Determination of the Acidity of the Soluble Starches

The acidity of the soluble starches was determined as pH and as titratable acidity. Titrations were made with 100 cc. of 2% soluble starch solutions (the equivalent of 2 g. of starch) using N/100 NaOH and 5 cc. of an 0.04% indicator solution. The pH determinations were made colorimetrically. The results of the acidity determinations are given in Table II. In this table the starches are arranged in the ascending order of their pH values.

From an examination of the data recorded in Table II it is clear that there is a wide range in the acidity values of the various starches, the pH values ranging from 3.3 to 6.3. Only the specially prepared starches had pH values close to the starch neutral point.

It should be pointed out that none of the commercial soluble starches met the accepted pH value of 4.5 to 4.7 as required by the

American Society of Brewing Chemists (1935) and the Association of Official Agricultural Chemists (1936) for the purposes of the Lintner determination. In fact, sample No. 1, which is an "approved" soluble starch, had a pH of 3.7.

TABLE II

ACIDITY OF SOLUBLE STARCHES AS pH AND AS TITRATABLE ACIDITY IN TERMS OF CUBIC CENTIMETERS OF N/100 NaOH. INDICATOR AS STATED

Sample number	pH value	Acidity to NaOH. Cubic centimeters of N/100 NaOH to alkalinity				
		Brom phenol blue [1]	Brom cresol green [2]	Methyl red [3]	Rosolic acid [4]	Methyl orange [5]
3	3.3	4.9	4.5	6.2	8.5	Neutral
4	3.3	4.0	4.5	6.1	8.3	Neutral
5	3.4	3.7	3.9	5.4	7.5	Neutral
2	3.5	2.4	2.2	3.9	6.0	Neutral
12	3.6	1.8	1.8	3.5	4.5	Neutral
1	3.7	1.3	1.7	3.0	4.8	Neutral
7	3.7	1.8	1.7	3.5	5.1	Neutral
10	4.5	.4	.2	1.7	4.6	Alkaline
11	4.5	.5	.2	1.4	2.2	Alkaline
9	5.0	Alkaline	Neutral	Neutral	2.0	Alkaline
6	5.9	Alkaline	Alkaline	.2	1.3	Alkaline
8	6.3	Alkaline	Alkaline	Alkaline	.8	Alkaline

[1] With brom-phenol blue titrations were made to a pH of 4.6.
[2] With cresol-green titrations were made to a pH of 4.7.
[3] With methyl-red titrations were made to a pH of 6.0.
[4] With rosolic acid titrations were made to a pH of 8.0±.
[5] No titrations were made with methyl-orange, end point inconclusive.

It is further evident from the data given in Table II that the value of any titration procedure for the determination of soluble starch acidity is of questionable value. Most any titration value can be obtained depending upon the choice of an indicator.

If a titration procedure must be used, however, it is believed that brom-cresol-green is the most suitable indicator. With this indicator the transition points are sharp and distinct. The color changes are yellow for acidity, green for neutrality, and blue for alkalinity. The indicator is apparently not affected by foreign substances present in the starch; neither is it affected by CO_2. Its neutral point is pH 4.6, practically the pH value for starch neutrality.

The relationship between the acidity of the soluble starches and their reducing power may be compared by examining the data recorded in Tables II and V. In a general way, those starches which are close to the neutral point (pH 4.6) have the lowest reducing powers. These observations appear to follow along with the statement made by Clark (1911) who wrote "The amount of reducing power of Lintner starch and the intensity of the erythrodextrin coloration with iodine solution

appears to be proportional, in a general way, to the acidity of the product." It should be immediately pointed out, however, that Clark's observations would not hold if in the preparation of the starches they had been carried beyond the soluble starch stage, and had been neutralized without purification of the final product.

Starch Susceptibility

To determine the susceptibility of the various starches to diastase a Lintner determination was made using each starch and a standard malt. The determinations were made by the Lane and Eynon (1928) method. The data are recorded in Table III. In this table the Lintner values are reported (1) in degrees Lintner without consideration of the reducing substances present in the starches, and (2) after deduction of the blank determination value to compensate for the reducing substances in the starches. For this purpose the following equation was used:

$$\frac{\text{Blank titration (cc.)} \times 4000}{(\text{Starch-infusion titration, cc.})^2} = {}^\circ\text{L.}$$

This may be simplified as follows:

$$^{(a)}\left[\frac{\text{Blank titer, cc.}}{\text{Starch infusion titer, cc.}}\right] \times {}^{(b)}\left[\frac{4000}{\text{Starch infusion titer, cc.}}\right] = {}^\circ\text{L.},$$

where a may be used as a factor.

For the purpose of eliminating the work incident to the making of a blank determination when the same starch is used, an attempt was made to develop a factor for this purpose. This factor was determined by dividing the observed Lintner value without reference to the reducing substances present in the soluble starch into the Lintner value after deduction of the blank determination. Obviously, the higher the factor the less will be the reducing substances present in the soluble starch and the more accurate the final determination. With a given starch the factor will remain the same as long as the final volumes of the hot titrated solutions are equivalent. The factors worked out for the various starches are shown in line 4 of Table III.

The results of the starch susceptibility study indicate that the starches were equally susceptible to diastase. The variation noted is due more likely to personal errors rather than to other contributory causes. The data also indicate that if confined to a single soluble starch, the use of a correction factor instead of continually running the blank holds promise.

Clarity of Solutions

Any solution of starch other than one described as opalescent would contain traces or more of insoluble starch. All the starch solutions

TABLE III

SUSCEPTIBILITY OF STARCHES TO DIASTASE

	Soluble starch number							
	1	3	6	8	9	10	11	12
Degrees Lintner								
Without correction	115.3	121.6	118.0	113.2	113.2	111.2	110.8	111.1
With correction	103.3	105.3	110.0	110.7	110.0	106.1	106.2	104.9
Difference	12.0	16.3	8.0	2.5	3.2	5.1	4.6	6.3
Correction factor	.896	.866	.932	.978	.973	.951	.955	.945

studied when treated with the buffer solution showed a tendency to cloud. On standing (one or more days) a sediment was observed in every starch solution. This may be considered as amylose as pointed out by Marquenne and Roux (1905). These authors observed that if a soluble starch solution was allowed to stand for several days under aseptic conditions that it gradually became opaque and deposits formed. This sediment was identified by them as amylose, the amylopectin remaining in solution. Data relative to the clarity of the starch solutions are shown in Table IV.

TABLE IV

CLARITY OF STARCH SOLUTIONS

Behavior of solution	Soluble starch number											
	1	2	3	4	5	6	7	8	9	10	11	12
Starch deposit	+	−	+	++	++	−	+	+++	++++	+	++++	+
Clarity of solution												
Opaque or opalescent	VSOq	Ol	Ol	SOq	SOq	Ol	VSOq	Oq	COq	SOq	COq	SOq
Degree of cloudiness	SC	FC	FC	C	C	FC	C	C	C	C	C	C
Impurities	−	−	−	++	−	+	−	−	−	−	−	−

VSOq = Very slightly opaque.
Ol = Opalescent.
SOq = Slightly opaque.
Oq = Opaque.
COq = Completely opaque.
SC = Slightly cloudy.
FC = Faintly cloudy.
C = Cloudy.

Reducing Substances in Soluble Starch

The reducing substances present in soluble starch are chiefly dextrins and some foreign material such as metals, the latter being introduced into the starch during the process of manufacture.

There has been considerable ambiguity in the classification of dextrins discovered and isolated by different analysts. For the pur-

poses of this investigation the dextrins formed during the hydrolysis of starch may be regarded as four in number. The first dextrin of conversion is amylodextrin. Amylodextrin gives a faint blue color reaction with iodine. The next dextrin of conversion is erythrodextrin. It gives a red color with iodine. The third dextrin of conversion is achroodextrin, and the fourth is maltodextrin. Neither the third or fourth dextrin gives an iodine color reaction. These dextrins are named in the order of lower to higher dextrins. The lower dextrins possess properties similar to those of starch with little or no reducing power, while the higher dextrins possess properties similar to those of the simple carbohydrates, as they have some reducing power.

According to the A.S.B.C. as well as according to the A.O.A.C., to be suitable for the Lintner determination a soluble starch must be *free from dextrin*, and contain not more than 0.50% of reducing substances calculated as maltose.

Determination of Reducing Substances

Determinations were made relative to the reducing substances present in the 12 samples of soluble starch under study. The results are recorded in Table II. The percentages of reducing substances reported were determined by the Munson-Walker method (see A.O.A.C. Book of Methods, 3rd Edition, page 379 (1930)) and are reported in terms of maltose, as well as dextrose. The Munson-Walker method was used because it is comparable to the Lane-Eynon method used in the Lintner determination and because it is more accurate than the Lane-Eynon method where minute quantities of reducing substances are present. Ost's or Bertrand's (see Browne, 1912) method would, of course, be even more preferable.

In order to insure the greatest accuracy when using the Munson-Walker method, the reduction of the Fehling's solution used was checked by standard dextrose and standard maltose solutions. The Fehling's solution values obtained were identical with those given in the Munson-Walker tables. The filtration of the cuprous oxide was made in Jena fritted glass filtering crucibles (type 1bG4).

From an examination of the data recorded in Table V, it is quite evident that there is an extreme variability to be found in the reducing substance content of soluble starches. This is particularly true of the commercial starches. In the commercial soluble starches the maximum and minimum amounts of reducing substances calculated as maltose are 13.94% and 0.97%. On the other hand, the reducing substances present in the specially prepared soluble starches were not in excess of 0.50% and in one sample the reducing substance content was less than 0.11%.

As a matter of interest it would appear as if the limitations for reducing substances in soluble starches as specified by the A.S.B.C. and the A.O.A.C. for the purposes of the Lintner determination were decided upon without too much attention being paid to the quality of the commercial starches available. Indeed sample 1, which was recommended as an approved starch by the A.S.B.C. "by a body designed for the purpose," contained almost a per cent of reducing substances calculated as maltose rather than the specified "less than 0.50%."

TABLE V

REDUCING SUBSTANCES IN SOLUBLE STARCHES

Reducing substances as	Soluble starch number											
	1	2	3	4	5	6	7	8	9	10	11	12
Dextrose %	0.630	3.845	0.942	4.250	1.280	0.535	9.06	0.260	0.168	0.250	0.320	0.310
Maltose %	0.970	5.916	1.450	6.540	1.980	0.829	13.94	0.403	0.109	0.388	0.496	0.480
Presence of erythrodextrin	+[3]	+[10]	+[4]	+[10]	+[3]	+	+[20]	+	+	+	+	+

Although the recently released methods for the analysis of malt as sponsored by the A.S.B.C. and the A.O.A.C. specify that the soluble starch to be used for the purposes of the Lintner determination shall contain *no dextrins*, no single soluble starch examined to date (particularly the commercial preparations) has been found to be free of dextrins. Each and every lot of soluble starch studied contained some erythrodextrin.

Discussion

The data shown in Tables I to V have been summarized as Table VI for ease of discussion.

By referring to the data recorded in Table VI it would appear that the specification set up by the A.S.B.C. and A.O.A.C. relative to the chemical characteristics of soluble starch for the purposes of the Lintner test are far too rigid in some respects and not sufficiently specific in other instances.

In only 2 of the 12 starches examined was the acidity as pH within the tolerance set and 1 of these was prepared from corn starch. None of the commercial soluble starches was ever close to the desired pH of 4.5–4.7. All starches, however, were easily buffered to a pH of 4.5–4.7.

No value appears to attach itself to the determination of acidity of soluble starch by means of titration methods. Most any value can be obtained depending upon the indicator used. If a titration method is

TABLE VI

SUMMARY OF SOME OF THE CHEMICAL AND PHYSICAL CHARACTERISTICS OF THE SOLUBLE STARCHES STUDIED

Type of soluble starch	Commercial Lintner Grade			Commercial Indicator Grade				Specially prepared				
Sample number	1	2	3	4	5	6	7	8	9	10	11	12
Moisture, %	11.13	16.90	3.27	13.75	12.48	14.88	11.75	9.81	5.88	12.18	9.48	6.80
Ash, %—Manufacturer's analysis	—	—	0.1	0.3	—	0.1	—	—	—	—	—	—
pH—2% solution	3.7	3.5	3.3	3.3	3.4	5.9	3.7	6.3	5.0	4.5	4.5	3.6
pH—2% solution—buffered	4.6	4.7	4.6	4.7	4.7	4.7	4.7	4.7	4.7	4.7	4.6	4.7
Acidity to (2 gms. (2% sol.) titrated with N/100 NaOH in cc.)												
Brom-phenol blue (pH 3.0 to 4.6)—to pH 4.6	1.3	2.4	4.85	4.0	3.7	Alk.	1.8	Alk.	Alk.	0.4	0.5	1.8
Brom-cresol green (pH 3.8 to 5.4)—to pH 4.7	1.7	2.2	4.5	4.5	3.9	Alk.	1.7	Alk.	N.	0.15	0.2	1.8
Methyl-red (pH 4.4 to 6.0)—to pH 6.0	3.0	3.9	6.15	6.1	5.4	0.2	3.5	Alk.	N.	1.7	1.4	3.5
Rosolic acid (pH 6.8 to 8.0)—to pH 8.0±	4.75	6.0	8.5	8.3	7.5	1.3	5.1	0.8	2.0	4.6	2.2	4.5
Methyl-orange (pH 2.9 to 4.0) expressed as neutral or alkaline	N.	N.	N.	N.	N.	Alk.	N.	Alk.	Alk.	Alk.	Alk.	N.
Reducing substances												
in terms dextrose, %	0.630	3.845	0.942	4.250	1.280	0.535	9.06	0.260	0.068	0.25	0.32	0.31
in terms maltose, %	0.970	5.916	1.450	6.540	1.980	0.829	13.94	0.403	0.109	0.388	0.496	0.480
Erythrodextrin-iodide index	6	60	22	60	10	1.25	100	1.67	1	1	1.25	1
Lintner determination												
With standard malt	115.3	—	121.6	—	—	118.0	—	113.2	113.2	111.2	110.8	111.1
With correction for presence of R. S.	103.3	—	105.3	—	—	110.0	—	110.7	110.0	106.1	106.2	104.9
Correction factor	.896	—	.866	—	—	.932	—	.978	.973	.951	.955	.945
Behavior of solution												
Unchanged starch	+	—	+	++	++	—	+	+++	++++	+	++++	+
Clarity of solution (opaque or opalescent)	V. sl. opaq.	Opal.	Opal.	Sl. opaq.	Sl. opaq.	Opal.	V. sl. opaq.	Opaq.	Comp. opaq.	Sl. opaq.	Comp. opaq.	Sl. opaq.
Degree of cloudiness (addition of buffer)	Sl. cl.	Faintly cloudy	Faintly cloudy	Cloudy	Cloudy	Faintly cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy
Impurities (cork, grit, fiber)	—	—	—	—	++	—	+	—	—	—	—	—

to be used, however, brom-cresol-green is the ideal indicator to use because its neutral point is pH 4.6.

With respect to diastatic susceptibility, all starches were apparently equally susceptible as no differences of note were experienced.

Phenomena which have not been given much attention are those of clarity and sedimentation. All of the soluble starch solutions produced a sediment, varying in this manner from lot to lot. It has been stated that this sediment is either amylose or unchanged starch. Sedimentation is important because starches that produce sediments, of course, do upset Kjeldahl's Law of proportionality, especially when such starches are used to determine the diastatic power of high Lintner value malt. Some statement, therefore, should be included in the specification regarding the clarity of the soluble starch solution and its tendency to sediment.

The presence of reducing substances in all the commercial grades of soluble starch was far in excess of the A.S.B.C. and the A.O.A.C.'s limitations (0.50% as maltose). The minimum amount found was 0.82%, the maximum amount 13.94%. The specially prepared starches only contained less than 0.50% reducing substances as maltose.

All starches contained dextrins in varying amounts, even the specially prepared lots. In fact, it is seriously questioned whether soluble starch can be made on a commercial scale that does not contain significant amounts of dextrin. In this connection, therefore, the limitations of the A.S.B.C. and the A.O.A.C. are too strict. Some maximum tolerance for dextrin content should be specified.

Supplementary Test For Determining Dextrin Content of Soluble Starch: the Erythro-dextrin Iodide Index

Inasmuch as soluble starch apparently cannot be prepared free from dextrins it becomes of value to know the approximate quantity of such substances present in soluble starch, especially in relation to the total amount of reducing substances. Some starches were examined which had a high reducing power but were low in dextrin content, whereas other starches were both high in dextrin content and high in reducing power. Obviously the two starches were not equally suitable for the purposes of the Lintner determination. In the first illustration, it is presumed that the high reducing power is due to foreign material introduced during processing. In the second example, the high reducing power for the major part is due to dextrins because tests for dextrose by the picric acid or orcinol test or with Nylander's reagent although giving positive reactions for dextrose indicated that dextrose was there in but small amounts.

If we assume the hydrolytic cleavage products of starch to be of the

order—starch, soluble starch, amylodextrin, erythrodextrin, achroodextrin, maltodextrin, isomaltose, maltose, and dextrose, it follows that if an analysis shows the presence of erythrodextrin, the presence of the higher dextrins and even maltose and dextrose must be assumed. Small (1919) has shown in his work that in the hydrolysis reaction that each cleavage product, as fast as it is formed, is itself a participant in a further reaction and that each step in the hydrolysis yields a product which is more easily hydrolyzed than its predecessor and that even some maltose and dextrose must form very early in the reaction.

With such information available, the suggestion immediately arises as to why an estimation of the erythrodextrin content of soluble starch cannot be used as a measure of its reducing substance content. Erythrodextrin gives a distinct wine red color reaction with iodine, whereas amylodextrin gives a faint blue color with iodine, and achroodextrin and maltodextrin give no color reaction with iodine.

Small (1919) has recommended a method for determining the presence of erythrodextrin in soluble starch. In his method, after precipitating the soluble starch, amylodextrins and undigested starch as starch iodide by means of a saturated ammonium sulphate solution, the precipitate is filtered off and the filtrate decolorized with dilute sodium thiosulphate.

To equal volumes of the filtrate and a control solution, equal quantities of a dilute iodine-potassium iodide solution are added. The erythrodextrin-red color stands out in contrast to the iodine color of the control.

It should be pointed out at this point, however, that the starch-iodide determination cannot be used for the quantitative determination of *starch*. According to Barger and Field (1912) the multiplicity of formulae (for starch-iodide) which have been suggested is in itself an argument against the correctness of any one of them. Barger and Field also hold the opinion that the blue substances of starch and iodine, when crystalline, may be the additive products of iodine in atomic proportion; but when they are colloidal and separate in the amorphous state, there is also a considerable adsorption of iodine, and, that the presence of electrolytes (KI) is necessary to bring about this adsorption by means of the cation. Kuster (1894) has shown that the iodine content of the "starch iodide" changes continuously with the concentration of the iodine solution with which it is in equilibrium; moreover, the ratio of partition between starch and water is not constant, but varies between wide limits.

An attempt was made by the authors to determine quantitatively the amount of erythrodextrin in soluble starch by a colorimetric procedure. This attempt, however, was not successful because of the

inability to prepare or obtain an erythrodextrin fraction of dextrin to use as a colorimetric standard.

It was found, after some trials, that a high quality dextrin could serve as a standard. Since dextrins, however, are complex mixtures depending on their source and method of preparation, one of the dextrins prepared from potato starch should be used. The dextrin selected as a standard should have a specific rotation between 175 and 185, with a reducing power of 8 ± (dextrose = 100). It should impart a wine-red color to iodine, have a pH value near that of neutral starch (pH 4.6) and be free from dextran or the "frog spawn" of the sugar industry.

Five dextrins were examined for these characteristics with the following results:

Dextrin origin	R.P. Dextrose = 100	pH 1% sol.	Appearance of solution
1. White (Potato)	10.30	3.8	Cloudy
2. White (Potato)	7.62	4.9	Clear
3. White (Corn)	12.35	3.5	Some dextran
4. Canary (Corn)	6.28	3.6	Amber
5. British Gum (Corn)	7.40	3.7	Amber

Dextrin No. 2 was selected as the standard as it more nearly approached the properties of the dextrins present in soluble starch.

Preparation of Standard Dextrin Solutions

Using sample of dextrin No. 2 as the standard dextrin, the following procedure was used for the purpose of preparing the standard dextrin solutions:

Five solutions of dextrin, 10 cc. in volume, but varying in concentration from 0.01% to 0.05% in 0.01% increments are prepared. Each solution is treated with 10 cc. of a 0.2% I − 0.6% KI solution saturated with $(NH_4)_2SO_4$ (Solution A of Small (1919)). The precipitate is filtered off by means of a No. 40 Whatman filter paper. If any blue particles are observed in the filtrate the filtrate is returned for refiltering. At this point the procedure deviates from that of Small's quantitative method in that no attempt is made to wash the precipitate with saturated $(NH_4)_2SO_4$ solution. Since the test is not strictly quantitative and it is desired to avoid any large and unnecessary increase in the volume of solution, 10 cc. of the filtrate are pipetted into clean test tubes and the iodine color eradicated with N/100 $Na_2S_2O_3$. This is done from a burette. The volume in the tube is then brought to a

volume of 19 cc. by means of distilled water. One cc. of a 0.3% I – 1.5% KI solution (solution B) is now added. These solutions which vary in dextrin content from 1 mg. to 5 mgs. serve as the standards against which the unknown solutions are later read.

Preparation of Soluble Starches for Dextrin Determinations

By means of a burette add to 10 individual test tubes the equivalent of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mgs., respectively, of the soluble starch solution whose dextrin content is to be determined. (On some occasions in order to attain very careful color comparisons, it may be necessary to prepare additional tubes of soluble starch.)

Bring the starch solutions to exactly 10 cc. with distilled water. Precipitate the starch as starch-iodide, filter, decolorize the filtrate with N/100 $Na_2S_2O_3$ and make to volume with I–KI solution and distilled water as described under "Preparation of Standard Dextrin Solutions."

Compare the intensity of the erythrodextrin red coloration in the soluble starch solutions with the color of the previously prepared dextrin standards by means of a Dubosque colorimeter. When using the Dubosque colorimeter if the cell of the colorimeter containing the standard dextrin solution is set on 2.0, the best results will obtain. The lower the reading of the soluble starch solution in comparison with the reading of the standard dextrin solution, the higher is the erythrodextrin content of the soluble starch solution being tested.

The dextrin content of the standard dextrin solution multiplied by 100, divided by the miligrams of soluble starch necessary to produce an erythrodextrin color equal to that of the particular standard dextrin solution becomes the erythrodextrin iodide index of the soluble starch under examination. The erythrodextrin iodide index therefore is an expression of the erythrodextrin in 100 mgs. of the soluble starch being examined.

The accuracy of the test depends upon the addition of an excess of iodine (Solution A of Small) to the soluble starch solution; clearing the filtrate free from fine particles of starch-iodide; using the exact amount of $Na_2S_2O_3$ to discolor the iodine in the filtrate; and, preparing the standard solutions of dextrin as well as the soluble starch test solutions to exactly the same volume (20 cc.). Differences of as little as 1 mg. of dextrin can be easily determined by the method.

The twelve soluble starches were analyzed in the above manner. Their erythrodextrin iodide index will be found in column 4 of Table VII. For comparative purposes the reducing power of each of the starches is given in column 6. Where the erythrodextrin iodine index is high the reducing power of the starch is likewise high. Also, where

the erythrodextrin-iodide index is high, maltose and dextrose are present. This can be confirmed by means of the picric acid, orcinol, or Nylander's reagent test.

TABLE VII

ERYTHRODEXTRIN-IODIDE INDEX OF THE SOLUBLE STARCHES

Starch number	Mgs. of dextrin in standard	Mgs. of starch to match standard	Converted to 100 mgs. starch basis	Erythro-dextrin-iodide index	Reducing substances as maltose
1	1	17	$\frac{1 \times 100}{17}$	6	0.97
2	3	5	$\frac{3 \times 100}{5}$	60	5.92
3	4	18	$\frac{4 \times 100}{18}$	22	1.45
4	3	5	$\frac{3 \times 100}{5}$	60	6.54
5	1	10	$\frac{1 \times 100}{10}$	10	1.98
6	1	80	$\frac{1 \times 100}{80}$	1.25	.83
7	2	2	$\frac{2 \times 100}{2}$	100	13.94
8	1	100	$\frac{1 \times 100}{100}$	1	.40
9	1	100	$\frac{1 \times 100}{100}$	1	.11
10	1	100	$\frac{1 \times 100}{100}$	1	.39
11	1	80	$\frac{1 \times 100}{80}$	1.25	.50
12	1	100	$\frac{1 \times 100}{100}$	1	.48

On the basis of the tests for erythrodextrin made so far, it is proposed that in addition to such properties of soluble starch as pH value and percentage of reducing substances, the dextrin content as measured by the erythrodextrin-iodide index be included and that any starch suitable for the Lintner value not have an erythrodextrin-iodide index in excess of 10.

Conclusion

Low ash content of soluble starch is indicative of low phosphate content. Low phosphate content appears to be desirable for optimum diastatic activity.

Freshly prepared soluble starch solutions should be prepared in

order to insure accurate results. Starches yielding cloudy solutions should be avoided as the suspended undissolved particles would interfere in any gravimetric determination. Precipitation of starch will also interfere with Kjeldahl's Law of Proportionality.

Reducing substances in soluble starch should not exceed 1%, in terms of maltose.

Soluble starch solutions should be at least alkaline to methyl orange, and acid to rosolic acid. However, the ideal condition would be neutral to brom cresol green.

The erythrodextrin-iodine index as described is an excellent supplementary test to indicate the quantity of soluble starch and serves also as a check on the reducing substances present in the soluble starch. The erythrodextrin-iodide index should not exceed 10.

Qualifications relative to the clarity of soluble starch solutions and the rate and quantity of deposition of unchanged starches should be included as specifications for suitable soluble starches.

The requirements of the A.S.B.C. and the A.O.A.C. for soluble starch for use in the Lintner determination are too severe as no soluble starch of commercial preparation has been found which will meet their specifications.

The properties associated with starch No. 6 indicate that it is the best type for use in the Lintner determination.

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IDENTIFICATION AND MEASUREMENT OF FACTORS GOVERNING DIASTASIS IN WHEAT FLOUR[1]

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(Read at the Annual Meeting, June 1936)

There has been considerable scientific inquiry as to fundamental causes for the wide variations in diastatic power that are exhibited by wheat flours when they undergo auto-digestion under comparable conditions—either in water suspensions or in doughs. Mangels (1926), Malloch (1929), Andrews and Bailey (1934), and others have shown that this diastatic activity is essentially a resultant of two main variables, namely, enzyme concentration and starch susceptibility. Mangels (1926) and Andrews and Bailey (1934) consider that starch resistance is the factor that is predominantly responsible for diastatic variations among flours.

The findings of Andrews and Bailey (1934) support the contentions of Nordh and Ohlsson (1932), Van Klinkenberg (1931), and others who state that the diastase of ungerminated seeds is almost exclusively beta-amylase, while that of germinated seeds is a mixture of both the alpha and beta forms. Waldschmidt-Leitz and Purr (1932), however, believe that alpha-amylase occurs in ungerminated barley, but in an inactive form, and that it requires the presence of "amylo-kinase,"—which occurs only in malted grain,—to become activated.

Those who believe that the amylase of ungerminated grain is almost exclusively beta-amylase—the saccharogenic amylase—have apparently drawn their conclusions very largely from the action of *water extracts* upon *soluble* starch. As will be shown later, however, the fact is that a very large portion of flour amylase is not extractable with water alone, nor can it be removed with 50% alcohol, which is sometimes recom-

[1] Published with the approval of the Director as Paper No. 180, Journal Series, Nebraska Agricultural Experiment Station.

mended for the purpose. Furthermore, the quantity of *soluble* starch occurring as a natural constituent of flour is relatively small.

The work of Józsa and Gore (1932) and of Gore and Józsa (1932) indicates that beta-amylase is not the only amylase occurring in ungerminated wheat flour. They find a considerable quantity of a starch liquefying enzyme, and they show that this enzyme is far more completely extracted from flour with salt solutions than with water alone. In applying to a group of flours a study of the action of their extracts on *soluble* starch, no apparent correlation between liquefying and saccharogenic power was found. This strongly suggests the presence of one or more amylases in addition to beta-amylase, although in view of the fact that here again soluble starch was the substrate, it was concluded that the value obtained had no direct bearing on the effective diastatic properties of flour.

More recently, Józsa and Johnson (1935) have revised Józsa and Gore's (1932) method for the estimation of liquefying power, using *soluble* starch as a substrate, and they now consider the method as a reliable means for determining alpha-amylase. If, indeed, alpha-amylase is the factor that is measured by this procedure, then it must be concluded that normal wheat flour contains alpha as well as beta-amylase.

This communication is a report of certain findings and conclusions resulting from studies initiated about two years ago in an attempt to secure additional information as to the number of amylases actively present in flour, and to make further inquiry into the matter of alleged variations in starch susceptibility. These studies involved different flour types, they have included both the water extractable and the water unextractable flour enzymes, and they have considered starches both in their natural and altered forms.

Preliminary Studies

For the purpose of investigating the properties of the starches and of the amylases, respectively and individually, it is highly desirable to have means for separating them from each other without altering the properties of either during the process of their isolation. In preliminary efforts to accomplish these things through the use of conventional and prescribed methods, the results obtained were generally uncertain, inconsistent, and entirely unsatisfactory.

Water extraction of amylase.—It is well known that when water extracts of different flours are allowed to act upon soluble starch, as in the Lintner type of method, the variation in saccharogenic power among the extracts is generally much less than when the same flours are sub-

jected to the autolytic method of the Rumsey type. Nevertheless, it was considered of interest to determine approximately how much of the active amylase is removed by water extraction, especially in view of the fact that most methods that have been suggested as suitable for the separation and idenification of amylases depend first of all upon their extraction with water or dilute alcohol.

It soon became evident that water fails to extract a large portion of the flour amylase. Moreover, considerable diastasis occurs during the extraction unless ice-water temperatures are maintained. Indeed, Blish, Sandstedt, and Astleford (1932) have found that even ice-water temperature does not completely inhibit diastasis.

A typical instance of the failure of water to extract all of the flour diastase is shown in the case of flour No. 5 (Table I) which had a maltose value of 258, as determined by the method of Blish and Sandstedt (1933). This flour was extracted with ice-water, centrifuged to remove the extract, again extracted with ice-water, and again centrifuged. The residue was then taken up with acetate buffer solution at a pH of 4.6 and autolyzed at 30° C. for 1 hour. It showed a maltose value of 137, indicating that even after two extractions with water it still retained more than one-half of its original diastatic activity. In another experiment involving repeated extractions of the same sample, there remained a maltose value of 60 after eight successive water extractions.

A series of experiments was undertaken for the purpose of comparing the activities of water extracts of flours on both raw and boiled wheat starch, and this study included flours varying over a wide range of diastatic activity. All but the durum wheat flour and the soft wheat flour were bakers' patent flours. Typical results are shown in Table I.

TABLE I

AMYLASE ACTIVITIES OF ICE-WATER EXTRACTS OF VARIOUS FLOURS ON RAW AND ON BOILED WHEAT STARCH

Flour	Maltose value	Raw wheat starch[1]	Boiled starch[2]
	Rumsey units	*Mg.*[3]	*Mg.*[3]
2	293	66	1738
5	258	68	2363
18	215	51	2056
26	177	50	2031
28	184	51	1811
32	228	57	1738
Durum wheat flour	500	51	3206
Soft wheat flour	102	52	3525

[1] 25 cc. of a 1 to 5 extract acting upon 5 g. of *raw* starch.
[2] One cc. of a 1 to 5 extract acting upon 2 g. of *boiled* starch.
[3] Milligrams maltose from 10 g. of starch incubated at 30° C. for 1 hour.

The data given in Table I show that the amylase extracted from flour by water is highly active on boiled starch, but that its activity on raw starch is slight in 1 hour. A noteworthy feature, however, is the fact that although the soft wheat flour had by far the lowest maltose value in Rumsey units, its water extract was far more active on boiled starch than were the extracts of any of the bread flours. In other words, the soft wheat flour had a much higher Lintner value than the others, although its ability to digest *its own starch* is very low. Soft wheat flours are recognized as being notoriously lacking in gassing power. This situation is further illustrated by another experiment in which water extracts of the soft wheat flour (Table I) and flour No. 2 (Table I), respectively, were allowed to act upon boiled starches from sources other than wheat. Data for this experiment are shown in Table II.

TABLE II

COMPARATIVE ACTIVITIES OF WATER EXTRACTS OF SOFT WHEAT FLOUR AND FLOUR NO. 2 ON BOILED STARCHES

	Extract of flour No. 2	Extract of soft wheat flour
	Mg.[1]	*Mg.*[1]
Activity on boiled corn starch[2]	1602	3721
Activity on boiled arrowroot starch[2]	1665	3745
Activity on boiled rice starch[2]	1273	3512
Activity on boiled potato starch[2]	1665	3354

[1] Milligrams maltose from 10 g. of flour incubated at 30° C. for 1 hour.
[2] 1 cc. of a 1 to 5 extract activity on 2 g. of boiled starch.

Table II shows that the soft wheat flour had a Lintner value of more than twice that of the No. 2 flour although the latter had a diastatic activity (by the autolytic method) of nearly three times that of the soft wheat flour. There are presumably at least two possible explanations for this situation. One obviously is that the starch of soft wheat is more resistant than that of hard wheat. The other is that the amylase of the soft wheat flour is almost exclusively beta-amylase—which attacks only soluble starch—whereas the hard wheat flour contains larger amounts of other amylases which are active upon raw starch.

Starch susceptibility.—There are at least two ways of accounting for variations in starch susceptibility as factors affecting diastasis when measured by the Rumsey auto-digestion method. One is to suppose that the entire starch portion of one type of wheat is intrinsically more resistant to diastatic attack than that of another. The other is to assume that flour starch is not strictly homogeneous, but that flours contain varying amounts of different fractions, and that these fractions, in turn, vary as to degree of susceptibility.

The complications involved in studying starch resistance in the light of these considerations are fairly obvious. The essential requirement is either to separate (in an unaltered form) *all* of a flour's starches, dextrins, *etc.*, from the accompanying amylase, or to find means for completely inactivating the flour amylase without either altering or removing any of the starch "fractions" during the process. Either of these operations has been found difficult to perform with entire satisfaction and confidence.

Methods of starch preparation based upon gluten washing, as used by Rask and Alsberg (1924), and by Mangels and Bailey (1933), are not suited to the problem at hand for at least two reasons, one being that considerable diastasis occurs while the dough is being prepared for the gluten washing process, and another being the removal of dextrin or other soluble fractions during the washing process itself. Either of these features tends to eliminate the more diastatically susceptible fractions originally present in the flour. Malloch's (1929) method for the inactivation of flour is open to a similar objection, since it involves centrifuging operations which also eliminate soluble fractions. Attempts to prepare and study starches from different flours by the foregoing methods including various modifications thereof, gave inconsistent and untrustworthy results.

Diastasis over Long Time Intervals

A possible basis for distinguishing and measuring inherent differences in starch resistance to diastase is afforded by studying rate of diastasis during autolysis over long time intervals. The feasibility of this type of method depends upon the following considerations:

1. In the early stages of flour auto-digestion—either in suspensions or doughs—maltose production is very rapid at first; later, as found by Landis (1934), it became a logarithmic function of time, finally diminishing to a stage where it is essentially a *linear* function of time. If sound flour (to which no malt preparation has been added) contains only beta-amylase, activity should practically cease as soon as all of the readily available starch has been converted into maltose, providing only such sound flour is used.

2. If, after the rate of maltose production has reached the rectilinear stage, some boiled or soluble starch is introduced, the rate of maltose production is at once enormously increased. We have found this to be the case for all flours, even for soft wheat flour of exceedingly low maltose value as measured in Rumsey units.

3. This shows beyond reasonable doubt that after the rectilinear stage has been reached there is always plenty of enzyme ready to use

up the starch as fast as it becomes available, and that from this point on, the rate of maltose production is in fact a quantitative measure of the rate at which the starch is being rendered available.

4. From these considerations, it follows that the angle of slope of the straight line graph (plotting sugar production against time) affords a quantitative expression of the rate at which the starch is being used up by the enzyme or combination of enzymes involved.

Figure 1 shows comparative rates of maltose production, for four flours of varying diastatic activities, over a period of 16 hours. Flour in buffered water suspensions were maintained at 30° C., and portions were withdrawn at regular intervals for maltose determinations by the ferricyanide method.

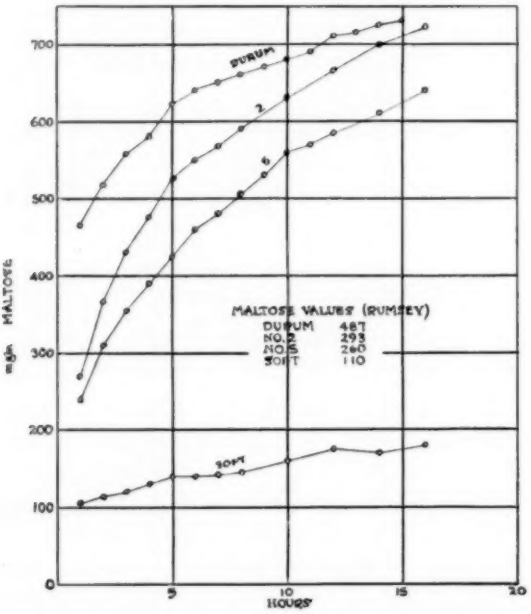

Fig. 1. Rate of auto-digestion by Ferricyanide method.

The curves shown in Figure 1 show a decided tendency to straighten out after diastasis has proceeded for several hours, and the slopes have different angles. The slight tendency for the curves to " fall off " in the later stages, instead of remaining straight (especially noticeable in No. 2 and No. 5), may conceivably be due to the fact that flour water suspensions do not remain homogeneous unless they are constantly shaken. We, therefore, undertook to employ a yeast-dough manometric procedure, using the " pressuremeters " described by Sandstedt and

Blish (1934). In this method, the rate of diastasis is recorded in terms of millimeters of pressure registered, over long-time intervals, by the carbon dioxide produced by the yeast fermentation of the sugars as they are liberated by the action of the amylase upon the starch. Here, instead of a flour-in-water suspension, one uses a more homogeneous medium, a slack dough or paste, containing 10 g. of flour, 7 cc. of water, and 0.3 g. of yeast. The pressuremeters are held at 30° C. and pressure is plotted against time. Figure 2 shows graphically a typical series of results obtained with a group of flours having a wide range of diastatic activities, as measured by the Rumsey type of method.

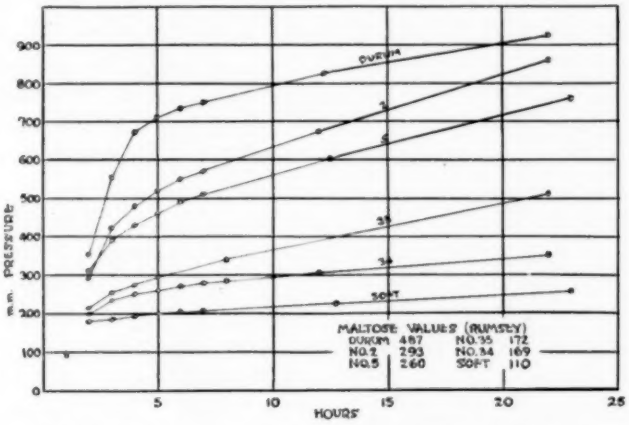

Fig. 2. Comparison of original flours by manometric procedure.

The curves shown in Figure 2 present a very clear picture of the comparative rates at which the greater portion of the flour starches, respectively, are converted into maltose during auto-digestion. From the very nature of the curves it is apparent that the manometric method not only confirms the results obtained by the ferricyanide method (Figure 1), but is decidedly a superior method for the purpose at hand, not only in convenience and simplicity, but also from the standpoint of giving sharply defined and clean-cut results.

Again it is seen that after several hours, all curves become essentially rectilinear, but have different angles of slope. With the exception of the durum flour, the degree of slope is directly proportional to maltose value, expressed in Rumsey units. These different degrees of slope seemingly are quantitative expressions of differences in rate of starch autolysis. If starch resistance is the sole factor that governs variations in flour diastatic activity, it might appear that here is a method for the quantitative measurement of differences in starch susceptibility, con-

fining ourselves, of course, to the bulk of the flour starch, and disregarding that portion that is very rapidly converted during the first stages of autolysis, before the rate becomes a linear function of time.

Enzyme Activity

The acceptance of these different angles of slope as quantitative expressions of differences in starch resistance must, however, rest upon the assumption that the enzyme factor is constant. If it is true, as reported by several investigators, that sound, ungerminated grain contains only beta-amylase, which is highly inactive on raw starch, one would expect that in the autolysis of sound wheat flour, saccharogenesis should practically stop after the more susceptible fractions of the starch, including dextrins, ruptured cells, *etc.*, had been digested.

Certainly, nothing even approaching this state of affairs is found in the cases of flours No. 2, 5, and 35. It is most nearly approached by the soft wheat flour. This strongly suggests that the flours contain, in addition to beta-amylase, at least one additional enzyme or activating substance that gradually renders the raw starch available to the beta-amylase which, as shown in Table I, is always present in an amount that is more than sufficient for the purpose. It, therefore, may be fairly concluded that the comparative degrees of slope are in reality quantitative expressions of relative amounts of this additional enzyme or activating factor itself. Is this factor alpha-amylase, is it an amylokinase, is it a cytase, or is it something else? Further work on the identity of this factor is in progress.

An examination of the foregoing conclusion is, of course, afforded by extracting the flour amylases quantitatively and allowing them to act upon the *same substrate,* which for our purpose should be raw, not soluble starch. As stated before, water extraction fails to accomplish any more than the partial removal of the total flour amylase. Józsa and Gore (1932), however, found the solubility of flour amylases to be much greater in salt solution than in water. We accordingly investigated the completeness with which flour amylase is extracted by various strengths of sodium chloride solution. The first step was to extract a high diastatic flour with salt solutions of several different strengths, and to test the activity of the residue after extraction.

Flour No. 2, having a maltose value of 293, was selected, and 20 g. portions were extracted for half-an-hour with 1, 2, 5, and 10% salt solutions, respectively, using 100 cc. of solution in each case. Each suspension was then centrifuged, and each residue was washed several times with more salt solution of the same strength as used in the original extraction. Each flour residue was then dried with alcohol and ether,

and subjected to the conventional autolytic test for diastatic activity, with results shown in Table III.

TABLE III

RESIDUAL DIASTATIC ACTIVITY OF FLOUR NO. 2[1] AFTER EXTRACTION WITH SODIUM CHLORIDE SOLUTIONS

Sodium chloride concentration	Residual activity
%	*Rumsey units*
1	46
2	38
5	24
10	18

[1] Original diastatic activity 293.

Table III shows that completeness of extraction is directly proportional to salt concentration, the 5 and 10% salt solutions apparently having extracted more than 90% of the total amylase.

The next order of procedure was to separate the amylase from the salt solutions by the addition of alcohol, and it was supposed that a final alcohol concentration of 85% by volume would be sufficient to affect complete precipitation of the enzymes. Accordingly, 75 cc. of each salt extract (equivalent to 15 g. of flour) were treated with enough alcohol to give the desired alcoholic concentration of 85%. The precipitate was allowed to settle overnight, most of the clear supernatant liquid was poured off and the precipitate with the remaining liquid was centrifuged and washed twice with alcohol. After completely decanting the alcohol from the centrifuge tube, the enzyme precipitate was taken up with acetate buffer (pH = 4.7) and made to 50 cc. 33⅓ cc. of this enzyme suspension (equivalent to 10 g. of the original flour) were then allowed to act upon 5 g. of raw commercial wheat starch for 2 hours at 30° C. The enzyme activities thus obtained are shown in Table IV.

TABLE IV

ACTIVITIES OF ENZYMES PRECIPITATED FROM VARIOUS SALT EXTRACTS WITH 85% ALCOHOL

Salt concentration used	Maltose from raw starch
%	*Mg.*[1]
1	284
2	274
5	218
10	84

[1] Milligrams maltose from 10 g. of starch incubated at 30° C. for 2 hours.

It would appear from Table IV that although the stronger salt solutions extracted the most enzyme, the activities of these enzymes after precipitation from 85% alcohol run decidedly in the reverse order. This suggested the possibility that the higher salt concentrations affected a considerable inactivation of the amylase, but such was found not to be the case. There then remained the possibility that 85% alcohol was not a high enough concentration to precipitate completely the amylase in the presence of the larger quantities of salt. This was investigated by repeating the previous experiments, but using higher alcohol concentrations. Typical data from these experiments are given in Table V.

TABLE V

EFFECT OF ALCOHOL CONCENTRATION ON COMPLETENESS OF PRECIPITATION OF AMYLASE FROM SOLUTIONS OF VARYING SALT CONCENTRATION

Salt concentration	Alcohol concentration	Starch converted
%	*% by volume*	*Mg.*[1]
1	80	284
1	90	304
5	80	196
5	90	302
5	91.6	306
10	80	84
10	90	256

[1] Milligrams maltose from 10 g. of starch incubated at 30° C. for 2 hours.

The values given in Table V show that the higher the salt concentration used for amylase extraction, the higher the alcohol concentration necessary to precipitate completely the amylase from the salt extract. As a result of the studies whose data are recorded in Tables III, IV, and V, it was deemed advisable to use 5% sodium chloride solutions for amylase extraction and 90% alcohol by volume for precipitation.

Using the foregoing procedure, then, the amylases were prepared from each flour of the series shown in Figure 2. Each enzyme preparation was allowed to act upon raw commercial wheat starch in the presence of yeast, and the respective rates of reaction were studied by the manometric method. A dough or paste was made which contained the amylase from 10 g. of flour, 0.3 g. of yeast, 10 g. of commercial wheat starch, and 7 cc. of water. Plotting pressure against time produced graphs shown in Figure 3.

The curves shown in Figure 3 substantially confirm the conclusion drawn from those that were presented in Figure 2. The degrees of slope for the different flours are in the same order in both series. It can only be concluded that those flours having the greater angles of

slope have correspondingly the larger quantities of the enzyme which is not beta-amylase, but which is capable of rendering raw starch susceptible to attack by beta-amylase.

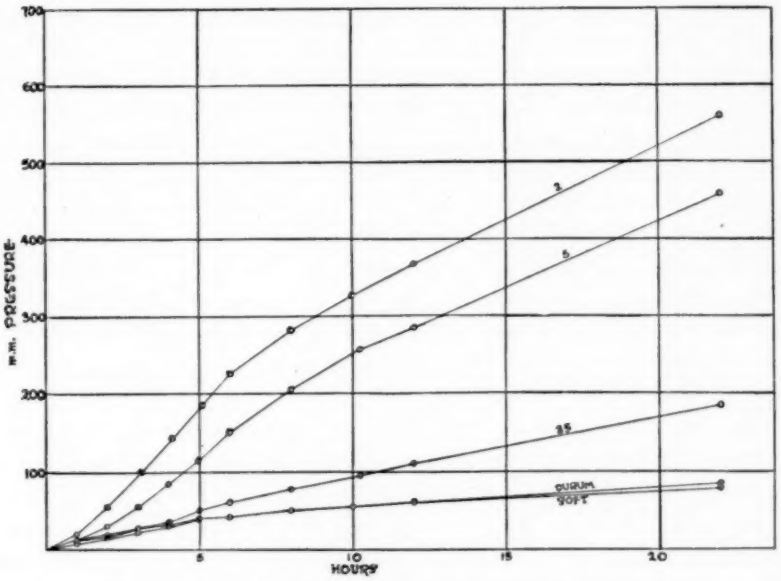

Fig. 3. Effect of NaCl extracts of flours on commercial wheat starch.

A conspicuous exception to the general rule is, however, the durum wheat flour, whose enzyme curve is almost identical with that of the soft wheat flour, despite the fact that the durum wheat flour has the highest maltose value (Rumsey units) and the soft wheat flour the lowest. The only reasonable explanation of this appears to be that the durum wheat flour contains a relatively very large fraction of starch in a form that is readily available for beta-amylase. Additional positive evidence of this will be shown later in this report.

Further Studies on Starch Resistance

As was previously mentioned, if one wishes to study, in a series of flours, the factor of comparative starch susceptibility, alone, it is necessary first of all to render the flour enzymatically inert, or to isolate all of the starch from each flour; in either case, one should use methods that do not significantly alter the starch properties. These starch substrates must then all be subjected to the same enzyme treatment under identical conditions. Certain difficulties encountered in this endeavor have been discussed.

In view of unsatisfactory experiences in attempting to use Malloch's (1929) recommended method for inactivating flour, the following procedure was adopted:

One part of flour was thoroughly shaken with 5 parts of 0.035 N hydrochloric acid, giving a pH of approximately 2, in order to inactivate the amylases as rapidly as possible. After standing for half-an-hour, the suspension was made a concentration of 80% alcohol, the added alcohol containing enough sodium hydroxide exactly to neutralize the acid. In this concentration of alcohol, all starch settles out, the supernatant liquid is removed, the material is finally treated with strong alcohol, and dried by air from a fan. The treatment accomplishes several things: it inactivates the flour, no starch is lost during the process, and all sugars originally present as such, are removed. It is, of course, possible that some starch is slightly altered by exposure to a pH of 2; nevertheless, all flours received the same treatment and the conditions are as strictly comparable as it has thus far been found possible to make them.

The comparative resistances of these inactivated flours were then studied, again using the yeast-manometric method under conditions as previously described. One series involved the addition of 0.2 g. of malted wheat flour to 10 g. of inactivated substrate, while in another series 0.1 g. of Taka-diastase was used instead of 0.2 g. of malted wheat flour. Typical results obtained are graphically presented in Figures 4 and 5, respectively.

From the graphs shown in Figures 4 and 5, it appears that the curves for the various flours show a pronounced tendency to parallel each other after having passed the earlier stage in which the more susceptible starch fractions were involved, and after the rectilinear stage is reached. Here, again, the durum wheat flour has shown exceptional behavior, which is explained readily enough by assuming that it contains a much larger quantity of the more susceptible starch fractions than the other flours.

The parallelism among the other inactivated flours indicates that in so far as the major portion of the flour starch is concerned, there were no significant differences in the starch resistances of the different flours.

From data thus far presented, it is apparent that beta-amylase is not the only enzyme concerned with diastatic activity in wheat flour. If the other factor involved is largely alpha-amylase, it also appears that in the series under consideration flour No. 2 contains the most alpha-amylase and the soft wheat flour the least. It should be possible to compare the relative amounts of this factor present in these two flours by allowing water extracts of flour No. 2 and soft wheat flour, respectively.

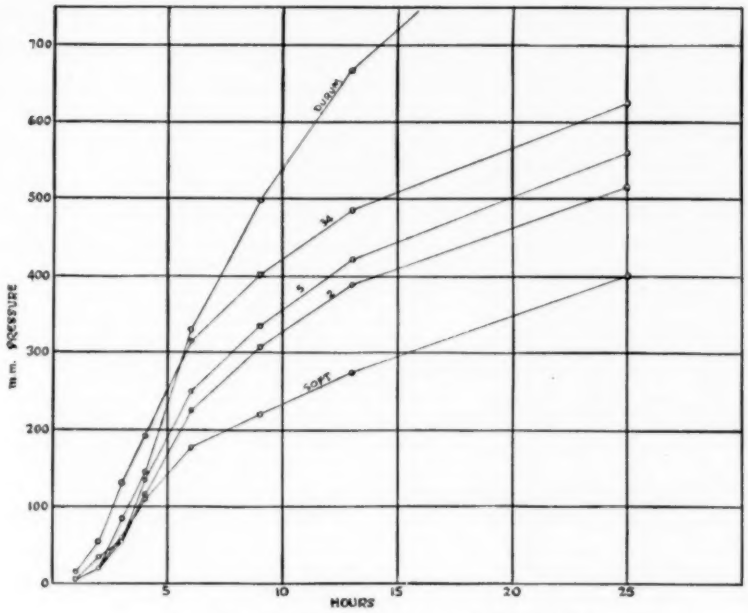

Fig. 4. Inactivated flours with 2% malted wheat.

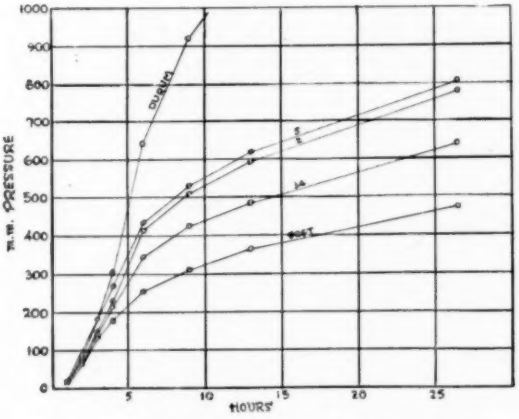

Fig. 5. Inactivated flours plus 1% Taka-diastase.

to act upon the inactivated flours. Confirmation is afforded in the event that the curves for the soft wheat extract are more nearly *horizontal* than those for the No. 2 flour. The flour extracts were prepared by extracting one part of flour with three parts of water, and in each case 10 g. of inactivated flour were made into a paste containing 0.3 g. of yeast and 7.5 cc. of extract (equivalent to 2.5 g. of flour). Rate of sugar production was followed by the manometric method, and the results are given in Figures 6 and 7.

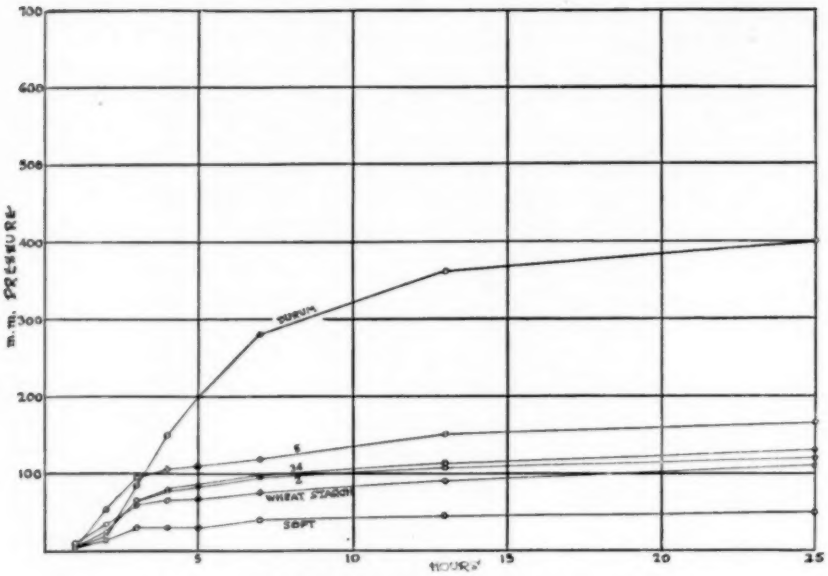

Fig. 6. Inactivated flours plus water extract of soft wheat flour.

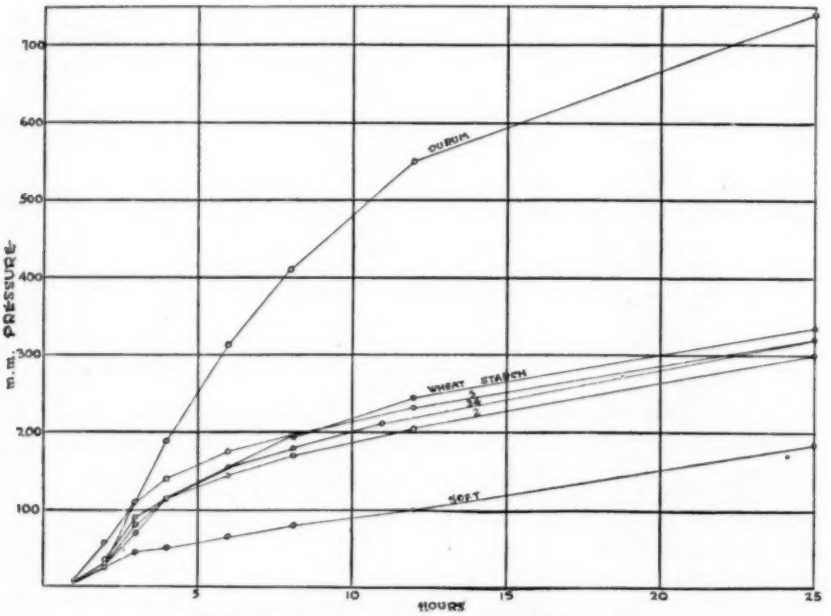

Fig. 7. Inactivated flours plus water extract of No. 2 flour.

The fact that the curves for the soft wheat flour extract acting upon the inactivated flours are nearly horizontal after the more susceptible portion of the starches have been used up (Figure 6), shows that the active substance must have been almost exclusively beta-amylase, which, of course, is unable to digest raw starch. The No. 2 extract, on the other hand, was able to act upon the raw starches at a very significant and steady rate, as shown by the angles of slope of the curves in Figure 7. In both cases, again, the curves tend for the most part to run parallel, showing insignificant differences in starch resistance in so far as the main portion of the flour starch is concerned.

Commercial vs. Experimentally Milled Flours

It has long been recognized that a commercially milled flour usually has a much higher diastatic activity, as measured in Rumsey units, than an experimentally milled flour from the same wheat. It was considered of interest to compare two such flours by the autolytic method over a long time interval, by the manometric method. The results of such a comparison are given in Figure 8.

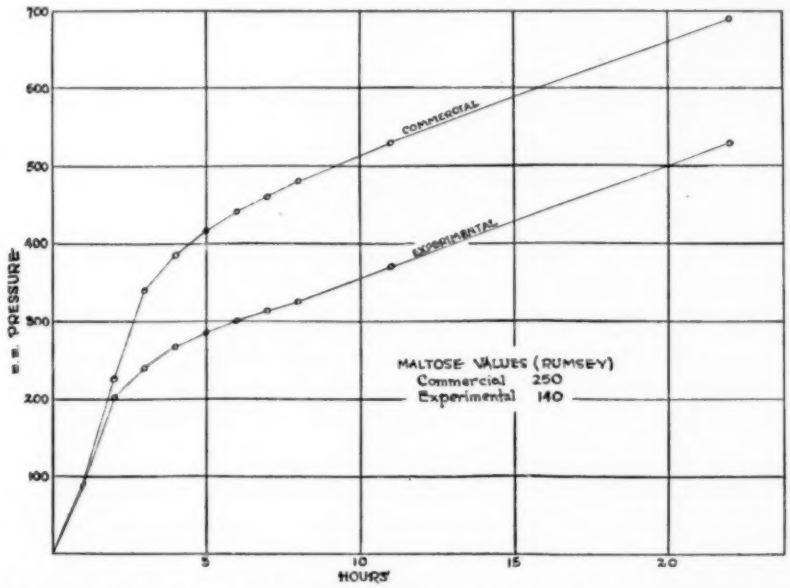

Fig. 8. Comparison of commercially and experimentally milled flours by manometric procedure.

The parallelism between the two curves in Figure 8, together with the fact that both curves show the same degree of slope is interpreted to mean that both flours contained significant and equal quantities of the

factor that digests raw starch and is not beta-amylase. These flours were made from supposedly sound wheat to which no malted wheat product had been added, which greatly minimizes the possibility that the presence of the additional enzyme might have been due to the presence of germinated wheat products.

It is concluded that, as compared with experimentally milled flours, commercial flours owe their higher diastatic activities to the fact that they contain relatively larger amounts of the more susceptible starch fractions—not to any differences in enzyme nature or content.

Diastatic Activity and Gassing Power

Occasionally a flour is found whose gassing power is higher than would be predicted from knowing its maltose value in Rumsey units, and its sucrose content. This may conceivably be due to a small quantity of readily available starch and an extraordinary high content of the enzyme that is responsible for rendering raw starch available, and which has been shown by these studies to be of general occurrence in sound wheat flour. The determination of maltose values by the Rumsey type of method involves only 1 hour's digestion. Properly conducted gassing power tests, on the other hand, involve much longer periods of time, which afford an opportunity for this enzyme to register its effects after the readily available portion of the starch has been largely used up.

From these considerations, it follows that where one wishes to study maintenance of gas production over fairly long time intervals, the manometric gassing power method is in every way preferable to the 1-hour maltose value test.

Summary and Conclusions

The average bakers' flour contains, in addition to an abundant supply of beta-amylase, at least one other enzyme or "activator" that affects rate of starch conversion in the auto-digestion of flour or dough. This may conceivably be "alpha-amylase," it may be "amylo-kinase," or it may be a cytase, or a combination of these factors.

The wide-spread belief that ungerminated grain contains *only* beta-amylase, in contrast to germinated or malted grain, which contains both the alpha and beta forms, may be an erroneous one.

Flours vary as to the quantity of this factor which they contain. Soft wheat flour apparently contains very little of it, and the same is probably true of durum wheat flour.

By far the greater portion of flour starch is "raw" starch, and unavailable to the beta-amylase that is so abundantly present, but all flours

contain very significant amounts of "available" starch as well. The "available" portion supposedly consists partly of ruptured or broken starch granules, and partly of starch that, due to structural peculiarities, is less resistant than the main portion.

Flours vary as to quantity of "available" starch. Durum wheat flour contains by far the most, and soft wheat flour, the least. The higher maltose value of commercial flour as compared with experimentally milled flour is probably due primarily to a difference in amount of ruptured starch granules.

With the possible exception of durum wheat flour and excluding the so-called "available" fractions, the unruptured starch granules of various flours are for all practical purposes identical in their resistance to attack by enzymes.

Maltose values, as registered by the 1-hour autolytic method, are apparently a resultant of two factors, one of which has to do with the amount and nature of the enzymes present, and the other of which is the quantity of the "available starch" fraction. After this fraction has been used up, however, the further rate of starch conversion depends exclusively upon the amount of the enzyme or activator which is not beta-amylase, but which renders the starch susceptible to the action of beta-amylase. This rate of "raw" starch conversion is a linear function of time, not a logarithmic function as was found by Landis (1934) to be characteristic of the stage involving the more susceptible portion of the starch.

The occurrence of this additional enzyme or activator explains why flours occasionally show greater gassing powers than would be predicted from a knowledge of their "maltose values" in Rumsey units.

The manometric method involving yeast—as herein outlined—is a rational, a convenient, and a trustworthy method for the study and measurement of factors herein discussed.

The enzymes that are chiefly concerned in the auto-digestion of flour starch may be almost completely and quantitatively extracted from flour by sodium chloride solutions, from which they may be precipitated with alcohol.

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STUDY OF SIZE OF BAKING PAN IN EXPERIMENTAL BAKING [1]

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(Read at the Annual Meeting, June 1936)

The Standard A. A. C. C. Basic Baking Test was primarily designed for hard wheat flours which find their utility in the commercial production of pan bread. Commercial doughs are generally handled at the peak of absorption and some supporting pan sides seem essential in handling such dough through the proving period and oven to obtain loaf uniformity and type of appearance expected in bread by the baker and his trade. The original conceptions of the small loaf experimental baking test described by Werner were in line with commercial practice. The Standard A. A. C. C. Baking Test for bread permits the adjustment of absorption to compare with commercial procedure. It therefore seems that the use of a baking pan in the experimental test is fully justified. However, for testing flour to be utilized strictly in hearth bread production the baking pan may be less essential or not desirable.

The tall- and low-form experimental baking pans described by Herman and Hart (1927) were designed so that a lengthwise slice of the small loaf would give something comparable to a commercial loaf slice when texture, grain, and crumb color were under examination. Numerous reports on small-loaf experimental baking tests made in these pans have been published in the last few years, and we find that test-bake operators in various parts of the country obtain considerable differences in loaf volume due to widely different flour characteristics and various combinations of ingredients, handling, and equipment. In the studies by Herman and Hart we find loaf volumes from 100 g. of flour averaging about 425 to 450 cc.; and in baking results reported by Canadian chemists (Geddes and Aitken, 1935) it is not uncommon to find loaf volumes ranging from 460 to 970 cc., averaging over 700 cc. Also it is not uncommon to find loaf-volume responses, such as reported by Blish and Sandstedt (1935), where a special treatment increased loaf volume 230 cc. or 51%. From the above figures we can see that a pan of one size has been expected to be the proper adjustment for the production of a large range of loaf volumes. The Stand-

[1] Subcommittee report, 1935–36 Committee on Standardization of Laboratory Baking.

ard A. A. C. C. Baking Test, in allowing a variable flour absorption, permits a variable dough weight to the pan. Instances of flours differing from 56% to 67% absorption are occasionally encountered, and we have a considerable difference in dough weight panned when 85 g. of flour dry matter forms the base for the dough. In a study of experimental baking pans, conducted by the Pioneer Section, A. A. C. C. (Davis, Leatherock, and Putnam, 1936), the comment of several laboratories was that the dough in the Standard A. A. C. C. Baking Test was too large for the pan. Pan No. 1 (modified low-form A. A. C. C. pan), studied in the above report, possibly gives more desirable loaf symmetry than the regular low-form A. A. C. C. pan, but it gives very little increase in pan volume and, therefore, does not correct this objection. To meet this objection, some laboratories, especially those working with higher loaf volume producing flours, scale their doughs down to obtain what they consider a better pan-dough relationship.

Laboratories experimenting with sponge doughs find very high loaf volumes when dough weights comparable to the straight dough procedure are panned. Here again doughs are often scaled down to give a more symmetrical loaf.

Pickering and Norton (1933) have very clearly shown that in commercial practice a definite size (approximately 5.8 cubic inches per ounce of dough) and shape of pan are best suited for the production of 16-ounce loaves. Communications from two large pan manufacturers agreed that the most popular size of pan supplied to the baking industry for the production of 16-ounce loaves was approximately 6.8 cubic inches per ounce of dough, or considerably larger than the pan found by Pickering to be best adjusted to this size dough or loaf. When we consider the size of the experimental pans (especially the low-form A. A. C. C. pan) in comparison to commercial conditions, it appears that the test baker is using a pan too small for the dough, while the commercial baker tends to use a pan larger than best suited to the dough for the 16-ounce loaf.

Since variable dough weight to pan is permitted by the Standard A. A. C. C. Baking Test in allowing absorption to be adjusted to suit the needs of the flour, and because doughs are often scaled to different weights in the actual practice of testing flours by the small-loaf procedure, we therefore find considerable differences in dough-pan relationships when the dough is panned. Some laboratories prefer to proof doughs for a definite time as specified in the standard procedure, and again this gives a varied dough-pan relationship at the time the dough goes to the oven, depending on the rate of gas production and gas retention during proof. Other chemists prefer to proof all doughs to a definite height or volume before placing in the oven in order to

obtain greater uniformity between replicates with reference to volume and loaf appearance. Automatic devices have been described by Harrel (1927) and Aitken (1930) to facilitate this procedure.

Experimental

In studying the effect of size and shape of baking pan in the baking of experimental loaves, it was thought advisable to vary dough weights to the tall- and low-form pans described in the Standard A. A. C. C. procedure to obtain different dough-pan relationships rather than to introduce pans of various dimensions. The different dough-pan relationships were studied principally to determine how they would affect specific loaf volume and the variability in loaf volume between replicated bakes, the interior and exterior type of the loaf, the differentiation between flours, and the measurement of dough response to an ingredient such as potassium bromate. Specific loaf volume in this study is the calculated loaf volume corresponding to 100 g. of dough.

Four dough weights were used, namely, 185, 165, 145, and 125 g., which correspond to approximately 95, 85, 75, and 65 g. of flour dry matter, and to approximately 4.4, 5.0, 5.7, and 6.6 cubic inches of pan space per ounce of dough for the tall-form A. A. C. C. pan, and 3.1, 3.45, 3.9, and 4.3 cubic inches of pan space per ounce of dough for the low-form A. A. C. C. pan.

Flours and formulae were selected so that the normal proof time of the 165-g. dough was 50 to 55 minutes, and the other doughs were proofed to the time required for the 165-g. dough to rise to normal proofing height. Other conditions of the baking test were essentially those specified in the Standard A. A. C. C. procedure and were so controlled that other factors than those being investigated would have a minimum of influence on the results.

Flour 2W was selected because of its green or unoxidized condition and because it gave a good response to the addition of potassium bromate to the formula. The bakings were conducted in the tall- and modified low-form A. A. C. C. pans. The baking results are recorded in Table I, and it will be observed that the specific loaf volume, variability between replicates, and response to 2.5 mg. of potassium bromate gave no definite differences due to the different dough weights. The highest dough weight, however, showed a tendency to give the greatest response to bromate. The exterior loaf type showed a decided change with a rugged break or development becoming more evident as the dough weight was decreased. This condition was most pronounced in the tall-form pan. The grain, texture, and crumb color remained constant in the various dough weights for either formula, the no-bromate loaves showed a dark yellow undeveloped characteristic and

TABLE I

THE EFFECT OF VARYING THE DOUGH WEIGHT TO THE A. A. C. C. EXPERIMENTAL BAKING PANS IN MEASURING THE BROMATE RESPONSE OF A FLOUR. FLOUR 2W

	Tall-form Pan				
	Dough weight from mixer—*Grams*				Average weight all doughs—*Grams*
	185	165	145	125	
No bromate					
Number of loaves	9	9	9	9	
Average loaf volume, *Cc.*	708.9	631.7	536.1	474.4	
Average specific loaf volume, *Cc.*	383.2	382.6	369.7	379.5	378.8
Standard Error of specific volume, *Cc.*	11.70	4.07	7.58	4.66	7.00
Coefficient of variation, %	3.05	1.06	2.05	1.23	2.09
2½ mgm. bromate					
Number of loaves	9	9	9	9	
Average loaf volume, *Cc.*	824.4	716.1	619.4	534.4	
Average specific loaf volume, *Cc.*	445.6	434.0	427.2	427.5	433.6
Standard Error of specific volume, *Cc.*	16.23	7.10	12.53	11.21	11.77
Coefficient of variation, %	3.87	1.63	2.93	2.62	2.76
Volume response, increase over no bromate loaves, %	16.4	13.4	15.4	12.6	14.4

	Low-form Pan (Modified, No. 1 Pioneer)				
	Dough weight from mixer—*Grams*				Average weight all doughs—*Grams*
	185	165	145	125	
No bromate					
Number of loaves	9	9	9	9	
Average loaf volume, *Cc.*	736.6	651.7	580.0	485.5	
Average specific loaf volume, *Cc.*	398.1	394.9	400.0	388.0	392.8
Standard Error of specific volume, *Cc.*	7.08	10.45	6.15	8.27	7.99
Coefficient of variation, %	1.77	2.64	1.54	2.12	2.02
2½ mgm. bromate					
Number of loaves	9	9	9	8	
Average loaf volume, *Cc.*	871.3	746.1	664.4	562.5	
Average specific loaf volume, *Cc.*	471.0	452.1	458.2	450.0	457.8
Standard Error of specific volume, *Cc.*	15.83	11.40	10.01	9.80	11.76
Coefficient of variation, %	3.4	2.52	2.19	2.17	2.57
Volume response, increase over no bromate loaves, %	18.1	14.4	15.0	15.8	15.8

the bromated loaves showed a bright-creamy slightly-overoxidized condition. The base of the no-bromate and bromated loaves remained about the same at the different dough weights except possibly the 125-g. dough loaves were a little smoother than those at the higher dough weights.

Flours 3D and 4D were selected because they were widely different in their commercial value. Flour 3D was a Southwestern baker's medium-extraction flour which had about 12% protein content and

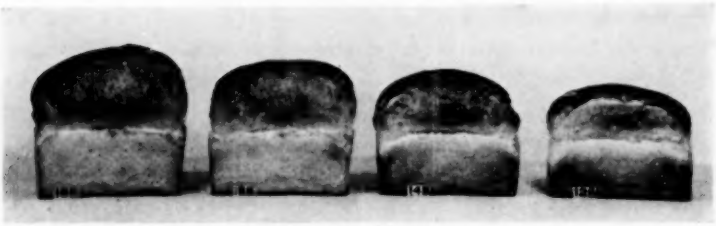
which gave excellent bakeshop results; while flour 4D was a long-extraction flour that had 10% protein content, low mixing tolerance, and in general would not be considered satisfactory for bakeshop purposes. Using the modified low-form A. A. C. C. baking pan, these flours were baked by one formula in a comparative manner to observe principally how the different dough weights affected the differentiation between these two flours. With the exception of grain, texture, and crumb color, we have recorded in Table II and Figure 1 the baking

TABLE II

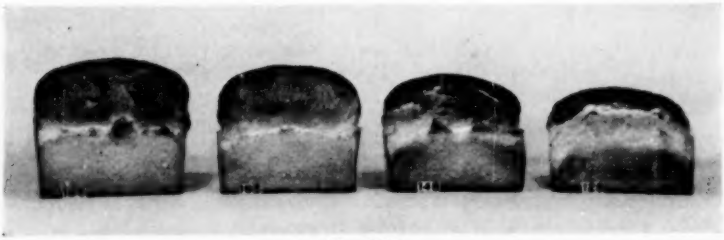
THE EFFECT OF VARYING DOUGH WEIGHTS TO THE MODIFIED LOW-FORM A. A. C. C. BAKING PAN ON FLOUR DIFFERENTIATION

	Dough weight to pan—*Grams*				Average weight all doughs—*Grams*
	185	165	145	125	
	Flour 3D				
Number of loaves	9	9	9	9	
Average loaf volume, *Cc.*	744.4	658.3	571.3	488.3	
Average specific loaf volume, *Cc.*	402.4	398.9	393.8	390.7	396.2
Standard Error of specific volume, *Cc.*	7.92	6.97	4.48	6.86	6.55
Coefficient of variation, %	1.96	1.75	1.13	1.75	1.46
Loaf type (Blish, 1928)	F	F	F−	G−	
	Flour 4D				
Number of loaves	9	9	9	9	
Average loaf volume, *Cc.*	669.4	593.3	527.2	455.0	
Average specific loaf volume, *Cc.*	361.8	359.5	363.6	364.0	362.4
Standard Error of specific volume, *Cc.*	4.64	3.04	6.22	4.36	4.56
Coefficient of variation, %	1.28	.84	1.71	1.16	1.25
Loaf type (Blish, 1928)	F	F−	G	H	
Loaf volume difference, flour 3D over 4D, %	11.2	10.6	8.3	7.0	9.3

results from these flours. There was a definite difference between the grain, texture, and crumb color produced by each flour, but the differences between the various dough weights for any one flour were not definite and would lead to the conclusion that dough-pan ratios did not affect these loaf characteristics when the doughs were otherwise handled the same. From Table II it will be observed that varying the dough weight to the pan did not show any definite tendencies in changing the specific loaf volume and loaf-volume variability between replicates. There was possibly a tendency toward increased loaf-volume differentiation between the flours in the higher dough weights and loaf-type differentiation was possibly greatest at the 145-g. dough weight. There was a very definite change in exterior loaf type as the dough-pan ratios were changed; the larger doughs were bold appearing with smooth break and shred; shell top tendencies increased as the dough weights decreased. This condition was most pronounced with

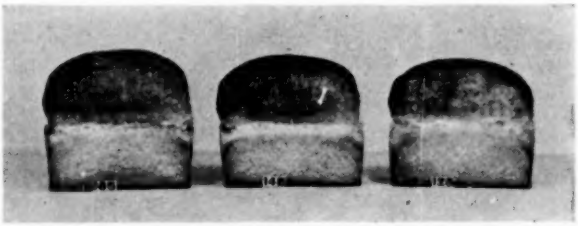


Flour 3D

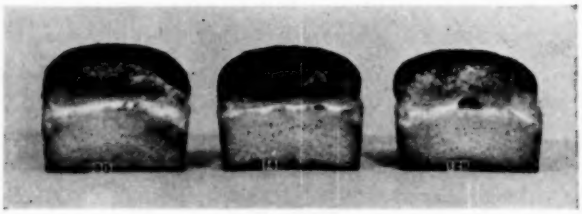


Flour 4D

Fig. 1a. Effect of varying dough weight to the modified low-form A. A. C. C. baking pan on the exterior of the loaf when doughs were proofed for the same time.



Flour 3D

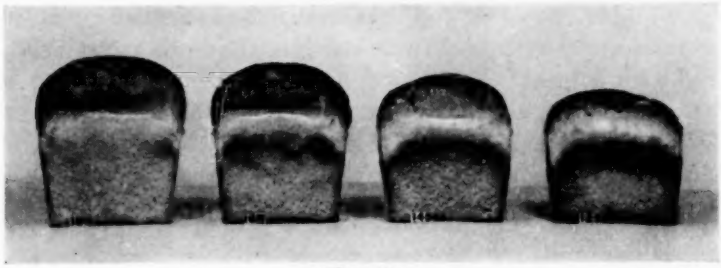


Flour 4D

Fig. 1b. Effect of varying dough weight to the modified low-form A. A. C. C. baking pan on the exterior of the loaf when doughs are proofed to same height.

flour D. The 125-g. dough weight loaves of either flour were so seriously shell-topped that differentiation between flours with respect to loaf type was difficult.

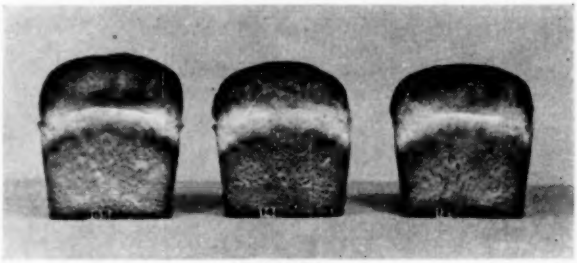
This series of bakes was also conducted in the tall-form A. A. C. C. pan. The variability of replicates was not measured but varying the dough weights had a pronounced effect on the type of the loaf as is



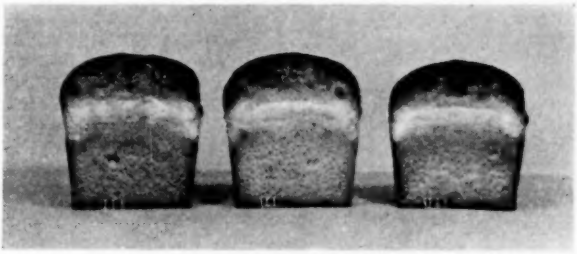
Flour 3D

Flour 4D

Fig. 2a. Effect of varying dough weight to the tall-form A. A. C. C. baking pan on the exterior of the loaf when doughs are proofed for the same time.



Flour 3D



Flour 4D

Fig. 2b. Effect of varying dough weight to the tall-form A. A. C. C. baking pan on the exterior of the loaf when doughs are proofed to same height.

shown in Figure 2a. The shell-top tendencies were more outstanding in the tall-form pan than in the low-form pan, and also more pronounced with flour 4D than with flour 3D. The interior of the loaves was not changed to any great extent by changing the dough weight. If any difference existed, the better grain and texture were found in the 185-g. doughs, but the differentiation between flours on this score was the same as at the other dough weights. There was a definite tendency for the base or the part in contact with the pan to become smoother as the dough weight decreased.

Collaborative Study

In order to be sure the various pan-dough relationships were not a result of some peculiarity of the oven condition used in one laboratory, a limited number of collaborators were called upon to measure some of the baking results likely to be affected in this study.

G. Moen, using his own baking formula and standard A. A. C. C. tall-form baking pans, studied the differentiation between two flours at the different dough weights. The results reported in Table III

TABLE III

EFFECT OF VARYING DOUGH WEIGHTS TO THE TALL-FORM A. A. C. C. BAKING PAN ON FLOUR DIFFERENTIATION.—(Collaborator G. M.)

	Dough weight from mixer—*Grams*				Average weight all doughs—*Grams*
	185	165	145	125	
	Flour 2M				
Number of loaves	10	9	9	10	
Average loaf volume, *Cc.*	613.	547.	488.	418.	
Average specific loaf volume, *Cc.*	331.	331.	336.	334.	333.
Standard Error of specific volume, *Cc.*	7.55	5.00	8.73	6.98	7.05
Coefficient of variation, %	2.28	1.51	2.60	2.09	2.12
Loaf type (Blish, 1928)	F	G+	H+	H+	
	Flour 3M				
Number of loaves	10	9	9	10	
Average loaf volume, *Cc.*	712.	621.	561.	478.1	
Average specific loaf volume, *Cc.*	385.	376.	387.	380.	382.
Standard Error of specific volume, *Cc.*	6.85	6.61	9.19	4.37	6.76
Coefficient of variation, %	1.78	1.76	2.39	1.15	1.77
Loaf type (Blish, 1928)	F	F	G+	H+	
Loaf volume difference, flour 3M over 2M, %	16.3	13.5	14.9	14.1	14.7

indicate that the specific loaf volume, loaf-volume variability between replicates, and differentiation with respect to volume were little affected by the different dough-pan ratios. Flour 2M produced loaves having a paler crust and darker crumb than flour 3M, and this difference

TABLE IV

EFFECT OF VARYING DOUGH WEIGHT ON BAKING BEHAVIOR OF FLOURS WITH RESPECT TO BROMATE RESPONSE IN THE LOW-FORM A. A. C. C. BAKING PAN.—(Collaborator W. F. G.)

	Malt-Phosphate Formula			
	Dough weight to pan—*Grams*			
	185	165	145	125
Flour 7G. Milled from Average No. 1 Manitoba Northern				
Number of loaves	4	4	4	4
Average loaf volume, *Cc.*	749.	648.	551.	475.
Average specific loaf volume, *Cc.*	405.	393.	380.	380.
External appearance [1]	3.5-g	3.0-g	2.5-g	2.0-g
Crumb texture [1]	5.0-c	5.0-c	4.5-c	4.5-c
Crumb color [1]	6.0-y	5.5-y	5.5-y	5.5-y
Coefficient of variability, %	.84	1.48	1.41	1.49
	Malt-Phosphate-Bromate Formula			
Number of loaves	4	4	4	4
Average loaf volume, *Cc.*	965.	844.	745.	605.
Average specific loaf volume, *Cc.*	522.	512.	514.	484.
External appearance [1]	4.0-O	4.5-O	5.0-O	3.5-O
Crumb texture [1]	7.0-O	7.0-O	7.0-O	7.0-O
Crumb color [1]	7.0-y	7.0-y	7.0-y	7.0-y
Coefficient of variability, %	2.12	2.79	1.45	.95
Volume response, percentage over no-bromate loaves	28.8	30.2	35.2	27.4
Flour 8G. Milled from Average No. 5 Manitoba Northern				
	Malt-Phosphate Formula			
Number of loaves	4	4	4	4
Average loaf volume, *Cc.*	702.	612.	546.	452.
Average specific loaf volume, *Cc.*	379.	371.	376.	362.
External appearance [1]	3.5	3.0	2.5	2.0
Crumb color [1]	3.0-c	3.5-c	3.5-c	3.5-c
Coefficient of variability, %	2.70	2.06	.88	2.12
	Malt-Phosphate-Bromate Formula			
Number of loaves	4	4	4	4
Average loaf volume, *Cc.*	776.	676.	576.	512.
Average specific loaf volume, *Cc.*	419.	410.	397.	410.
External appearance [1]	4.0-O	3.5-O	3.0-O	2.5-O
Crumb color [1]	4.5-c&o	5.0-c&o	5.0-c&o	5.0-c&o
Coefficient of variability, %	.97	1.11	1.30	.98
Volume response, percentage over no-bromate loaves	10.5	10.4	5.5	13.3
Average bromate response for the two flours, %	19.6	20.3	20.3	20.4

[1] g = underfermented characteristics
O = overfermented characteristics
External appearance = (Perfect score 5.0)
Crumb texture = (Perfect score 10.0)
Crumb color = (Perfect score 10.0)
c = coarse; g = grey; o = open; y = yellow.

TABLE V

EFFECT OF VARYING DOUGH WEIGHTS TO THE TALL-FORM A. A. C. C. BAKING PANS ON FLOUR DIFFERENTIATION.—(Collaborator A. A. A.)

	Dough weight to pan—*Grams*				Average weight all doughs—*Grams*
	185	165	145	125	
	Flour W				
Number of loaves	9	9	9	9	
Average loaf volume, *Cc.*	739.4	632.7	546.6	464	
Average specific loaf volume, *Cc.*	399.7	383.5	377.0	371.2	382.8
Standard Error of specific volume, *Cc.*	10.25	9.16	8.48	8.30	9.05
Coefficient of variation, %	1.38	1.44	1.55	1.79	1.54
Loaf type (Blish, 1928)	F	F	F	H+	
	Flour F				
Number of loaves	9	9	9	9	
Average loaf volume, *Cc.*	659.4	600	518.3	450.5	
Average specific loaf volume, *Cc.*	356.5	361.6	357.4	360.4	358.7
Standard Error of specific volume, *Cc.*	12.16	16.58	8.83	9.22	9.20
Coefficient of variation, %	1.84	2.73	1.70	2.05	2.08
Loaf Type (Blish, 1928)	F	G	H	H−	
Volume difference, percentage over flour W	12.1	6.05	5.5	2.9	

appeared constant in all loaves. The grain and texture held constant throughout all loaves, although there was a substantial difference in the texture between the two flours. The exterior loaf characteristics were definitely affected by the different dough weights as indicated by reference to the types described by Blish (1928).

W. F. Geddes, using flours of high and low response to potassium bromate and a malt phosphate formula, varied the dough weights to the standard low-form A. A. C. C. pan, and made the observations recorded in Table IV. No consistent difference was found in the variability between replicate loaf volumes. A tendency for higher specific loaf volume at the higher dough weights was considered contrary to the idea that larger doughs should give lower volume due to "crowding" in the pan. Dough weight had no material effect on bromate response with reference to volume and the appearances of age. The average external appearance score decreased with decreasing dough weight due principally to the general shape of the loaves (symmetry and boldness), break, and shred. The color of the crumb was not affected by varying the weight of the dough. The 165-g. doughs with strong Canadian flours, and a malt phosphate bromate formula, was thought to give unshapely, top-heavy loaves with too much of the loaf out of the pan. Either 145 g. or between 145 and 165 g. of dough was considered to be the most suitable for this pan, formula, and type of flour in order to obtain the most satisfactory test loaves.

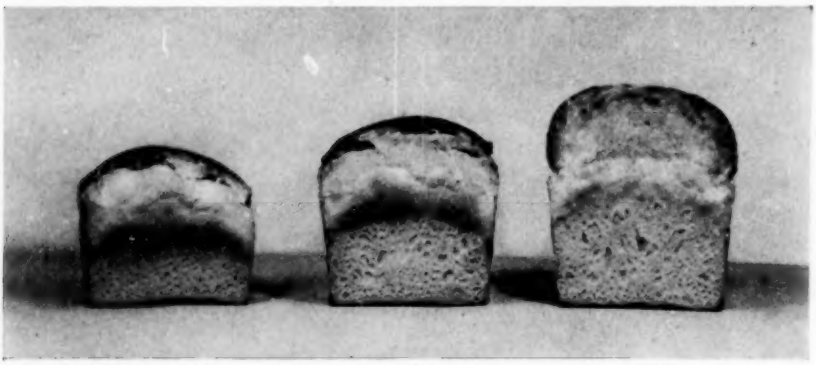
A. A. Andre selected two flours having a very definitely different commercial evaluation and using a modification of the standard baking test differentiated between the two flours at various dough weights. The specific volume showed a definite increase for flour W, but not for flour F, as the dough weight was increased in the pan. Variability between replicates showed no marked tendencies, due to the different dough weights. The inside loaf characteristics of each flour were constant at all dough weights. The differentiation between the flours with respect to volume difference was considerably greatest at the 185-g. dough weight. The 165- and 145-g. doughs, however, gave about the same differentiation. The external loaf type was definitely changed by changing the dough weights; this was most pronounced with flour F. The best differentiation with respect to external loaf type appeared at the 145-g. dough weight.

Proofing to Constant Height

In these studies in which the dough weight was varied to the tall- and low-form A. A. C. C. pans and all doughs proofed for the same time, a definite difference in type of loaf exterior was the outstanding loaf characteristic affected. When the loaf type as described by Blish (1928) is used as the criterion in interpreting the baking test results and obtaining an estimate of the nature or condition of the dough from a flour, it is necessary to know to what extent this exhibition is a result of the nature or condition of the dough and to what extent it might be an exhibition of dough-pan relationship in the baking reactions.

From experience in varying the dough weight to baking pans, indications were that the relationship between the height of the dough in relation to the top of the pan at the time the dough was placed in the oven was a critical factor in establishing the exterior type of the loaf. Flours 3D and 4D, which had been proofed for a definite time at the different dough weights, were proofed to the same height (Figures 1b and 2b) and it was found that the increased shell-top condition which came with decreased dough weight to pans disappeared. The longer proof, however, gave a less desirable loaf interior and increased the specific loaf volume.

Flours which are deficient in diastase and baked on a no-sugar or low-sugar formula are generally slow in the proof and shell-top loaves are characteristic. This is sometimes considered an exhibition of the low proteolytic activity accompanying the low diastatic activity (Figure 3). Such characteristics, however, can be entirely changed in the loaf if sufficient dough is panned. In this instance 130, 165, and 200 g. of dough were panned in the tall-form pan, and the 200-g. dough which



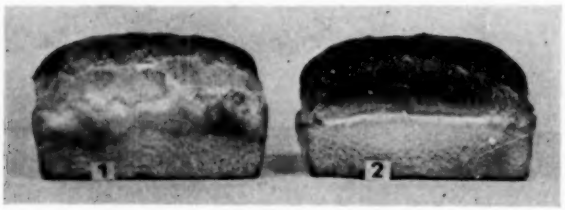
Grams of dough....130 165 200

Fig. 3. Effect of varying dough weight of a sugar-deficient dough to the tall-form A. A. C. C. baking pan and proofing to time.

proofed to the normal proofing height gave a bold, smooth break in contrast to the shell-top appearing loaves from the lower dough weights which did not proof to the top of the pan. Here, again, the relationship of the top of the dough to the top of the pan at the time the dough goes to the oven is a critical factor in establishing the exterior type of the loaf, making it difficult to obtain a true loaf estimate of a condition or nature of the gluten in the baking reaction.

Several attempts had been made to utilize in the small loaf baking procedure a pan used by bakers for the production of small sample loaves. The dimensions of this pan are: top, $5\frac{1}{2} \times 3$ inches; bottom,

Flour 3D



Flour 4D

(1 and 3 special pans. 2 and 4 special pans, depth reduced $\frac{1}{2}$")

Fig. 4. Effect of changing the depth of a special type baking pan on loaf exterior using 165 g. dough.

5 × 2½ inches; and depth, 2¼ inches, and when 165 g. of dough is placed in this pan, the dough-pan ratio is 5.2 cubic inches of pan space per ounce of dough, or considerably less space than the optimum dough-pan ratio used in the bakeshop. These attempts resulted in an inferior shell-top appearing loaf from doughs which gave loaves of very good appearance using the A. A. C. C. experimental pans. This sample loaf pan was cut down to ½ inch less depth and the improvement in the loaves from flours 3D and 4D is shown as Figure 4. In this instance where a dough from 200 g. of flour was divided and each part proofed for the same time and baked in pans which were different only in the relationship of the top of the proofed dough to the top of the pan, we cannot say that the widely different exterior loaf types can be considered entirely a measure of the nature or condition of the dough. In this study where the size and shape of the pan were modified to obtain a different relationship of the top of the proofed dough to the top of the pan, we have evidence supporting the data obtained by varying the dough weight in the A. A. C. C. experimental pans in which this dough-pan relationship was studied and found to be a critical factor in establishing the exterior type of the loaf.

Summary and Conclusion

A study was made of the influence of the size and shape of the baking pan in experimental baking by panning 185, 165, 145, and 125 g. of dough to the tall- and low-form A. A. C. C. baking pans. This procedure was followed in studying various dough-pan relationships in preference to introducing different pan sizes and shapes.

When all dough weights were proofed to the same time, the following baking results were not definitely affected by these different dough-pan relations: specific loaf volume; variability in loaf volume between replicates; response to potassium bromate; loaf interior characteristics as texture, grain and crumb color; and differentiation between flours with respect to volume and inside characteristics.

The exterior loaf characteristics were definitely affected by the different dough-pan relations, bold break and smooth shred being characteristic of the 185-g. dough weight and shell-top characteristics increasing with decreasing dough weights. This shell-top condition was most prominent in flours which gave lower specific loaf volume and was less pronounced in the low-form pans than in the tall-form pans. These results were drawn from studies in different laboratories using different flours and various formula modifications. The external loaf characteristics were not definitely affected when the different dough weights were proofed to the same height. *The relation of the top of the dough to the top of the pan at the time the dough goes to the oven*

was found to be a critical factor in establishing the external type of the resulting loaf.

In classifying a flour according to the external type of loaf it will produce, it appears that the doughs should be proofed to a definite height or volume so that dough-pan relationship at the time the dough goes to the oven will be constant and not be a serious factor in establishing the exterior loaf characteristics. When the rate of gas production in proofing varies between two flours and they are proofed for the same time in a comparative bake, the difference in exterior loaf type should not be seriously considered as an index of the nature or condition of the dough.

In a summary of all factors studied, dough weights from 145 to 165 g. in the standard A. A. C. C. baking pans proofed to a definite height or volume gave possibly the best dough-pan relationship. If a standard quantity of dough to the pan is not a standard practice, then the loaf volume should be expressed in the baking report as specific loaf volume, that is, the calculated cubic centimeters loaf volume from 100 g. of dough.

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REPORT OF THE U. S. DEPARTMENT OF AGRICULTURE BREAD FLAVOR COMMITTEE

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and

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(Read at the Annual Meeting, June 1936)

The Department of Agriculture for many years has been studying various problems related to bread. These studies have been undertaken in three bureaus—the Bureau of Agricultural Economics, the Bureau of Chemistry and Soils, and the Bureau of Home Economics, each with its own point of view.

During the summer of 1935 there were several conferences of members of these various bureaus of the Department with representatives of the milling and baking industry, for the purpose of integrating efforts of the Department and the various industrial groups. A committee on bread studies was appointed within the Department, with Assistant Secretary M. L. Wilson as chairman. Since it was the consensus of opinion that a thorough study of the factors that enter into bread flavor was needed, a subcommittee was appointed consisting of D. A. Coleman, Bureau of Agricultural Economics, J. A. LeClerc, Bureau of Chemistry and Soils, and F. B. King, Bureau of Home Economics, chairman. It was agreed that a cooperative study of bread flavor would be undertaken to determine the influence of the various constituents entering into bread under controlled processes.

Surveys have been undertaken by baking and allied industries to determine consumer preference on such merchandising factors as size and shape of loaf, effect of slicing, thickness of slice, effect of wrapping, and freshness. Other surveys have been undertaken to determine consumer preference judged by the amount and kinds of bread consumed in a given area.

A second type of investigation attempts to determine the relative effects on bread flavor of variations in formula, procedure, and mer-

chandising. Samples of bread are prepared varying one factor at a time, and these samples are submitted in series to a number of judges for indication of preference. The resulting data are analyzed statistically to evaluate the method used in the study and the significance of the results. A study of this kind has been reported by Ingels, Irwin, and Landis (1936). Under stimulus of the committees on bread flavor and consumption from the allied industries, projects are being carried on cooperatively by many of the bakers.

A third type of study attempts to identify by chemical analyses the substances entering into bread flavor. This method is fairly new for the bread industry, but it has been used for many years and still is practiced as a means of improving flavor in beer and wines. It is the type of study carried on by Visser't Hooft and de Leeuw (1935) in this country and by Maiden (1936) in England.

The committee of the Department of Agriculture decided to use the second type of study, starting with a series of tests with flours made from known samples of different classes of wheat. After determining the judges' preference for bread made with wheat of a given class, the next test would be to determine preference as to the grade of flour from the preferred wheat. Using the preferred wheat and the preferred grade of flour from that wheat, the bleaching of the flour would be the next variable in the tests, and so on.

All of the bread was made in the laboratories of the Bureau of Agricultural Economics under conditions approximating commercial procedure. To date the following factors have been studied in duplicate:

1. Class of Wheat—
   - Soft red winter
   - Hard red winter
   - Hard red spring
2. Grade of Flour—
   - Straight
   - 85% patent
   - 70% patent
3. Bleaching of Flour—
   - Unbleached
   - No. 1 bleach
   - No. 2 bleach
4. Fermentation and Baking Temperature of the Dough—
   - Ferment 86° F., bake 450° F.
   - Ferment 86° F., bake 425° F.
   - Ferment 78° F., bake 425° F.
5. Type of Formula—

	Lean	Medium	Rich
Absorption	57.8%	61.8%	63.2%
Sugar	3.5	3.5	5
Salt	1.5	1.5	1.5
Yeast	3	3	3
Fat	—	3	5
Milk powder	—	4	6
Flour (15% moisture basis)	300 g.	300 g.	300 g.

6. METHOD OF MIXING—
   Sponge
   Straight dough
7. FRESHNESS OF BAKED BREAD—
   12, 24, 48 hours old

Procedure

Except when the three formulas themselves were compared, all the bread was made by the medium formula. The straight-dough method was employed, and the dough was fermented at 86° F. and baked in single pound-size pans at 450° F. The loaves were allowed to cool for about 1 hour, then wrapped in heavy waxed paper, and stored at room temperature until the next morning. Except when freshness was the factor being studied, the bread was sampled when it was approximately 24 hours old.

Serial No.

READ BEFORE JUDGING

Determine odor first, smelling the lowest numbered sample first and the highest numbered sample last—the same for taste. Rinse mouth with water before tasting each sample.

	Sample number	
	Odor	Taste
Best		
Intermediate		
Poorest		
Excellent		
Good		
Fair		
Poor		

Order of desirability
First Second Third

Name.....

Date.....

Return sample numbers with score cards.

Figure 1. Sheet for recording judgments on the bread samples.

The bread was sliced by a commercial slicing machine and test samples consisting of two adjacent slices were placed immediately in glassine bags, bearing identification numbers. The top of each bag was folded down and fastened with a clip. Except for tests to compare the relative merits of straight or sponge doughs, 3 samples were

TABLE I

NINETY-SIX JUDGMENTS IN SERIES OF FLAVOR TESTS ON BREAD, EXPRESSED IN PERCENTAGE FOR THE SEVEN FACTORS TESTED

Factor	Test	Odor		Taste	
		Best	Poorest	Best	Poorest
		%	%	%	%
Class of wheat					
Soft red winter	1	30	36	41	37
	2	17	59	24	56
Hard red winter	1	25	37	23	31
	2	38	24	27	27
Hard red spring	1	45	26	36	31
	2	45	17	49	17
Grade of flour					
Straight	1	20	44	20	41
	2	35	41	28	44
85% patent	1	41	26	44	26
	2	36	25	39	21
70% patent	1	39	30	37	33
	2	28	33	32	35
Bleaching of flour					
Bleach No. 1	1	26	30	27	34
	2	29	35	29	27
Bleach No. 2	1	37	35	41	32
	2	38	30	38	31
Unbleached	1	37	35	32	32
	2	33	35	32	43
Type of formula					
Lean	1	15	56	14	64
	2	11	58	13	56
Medium	1	35	24	34	20
	2	32	29	31	29
Rich	1	50	20	52	15
	2	57	12	55	14
Method of mixing					
Straight dough	1	51	49	42	57
	2	37	62	27	72
Sponge	1	49	51	57	42
	2	62	37	72	27

TABLE I—*Continued*

Factor	Test	Odor		Taste	
		Best	Poorest	Best	Poorest
		%	%	%	%
Fermentation and baking temperatures					
Ferment 86° F. Bake 450° F.	1	31	38	28	44
	2	14	55	18	51
Ferment 86° F. Bake 425° F.	1	28	36	34	30
	2	51	16	48	17
Ferment 78° F. Bake 425° F.	1	41	26	38	25
	2	35	29	34	32
Freshness of baked bread					
48 hours old	1	31	40	25	46
	2	30	38	24	51
24 hours old	1	22	38	30	34
	2	25	29	35	21
12 hours old	1	47	22	44	20
	2	45	33	41	28

compared, so these samples were placed in a larger container and in this form given to the judges.

The testing of individual samples was staggered in the same manner as reported last year by Ingels, Irwin, and Landis (1936), in which 6 judges give a complete coverage of arrangement of order of judging 3 samples. The bread samples were delivered to the desks of the judges shortly before lunch time.

There were 96 judges gathered from the three bureaus represented on the departmental committee. They included executives, scientists, laboratory technicians and aides, stenographers, and clerks, and came originally from all parts of the United States. As a group they are probably representative of consumers in general, though some have had considerable experience in judging the quality of food products including bread.

The score card used for rating the bread is shown in Figure 1.

Results

The decisions of the judges for each test as summarized in Table I were grouped in a frequency table giving the distribution of each kind of bread and the rank as "best," "intermediate," and "poor." The value of Pearson's X^2 was calculated, and Fisher's (1932) X^2 table was used to test whether or not the decision of the judges was significant.

CLASSES OF WHEAT. Commercial lots of soft red winter, hard red winter, and hard red spring wheat that enter into commercial channels, were milled into 95% patent unbleached flour in the laboratories of the Bureau of Agricultural Economics and made into bread using a medium commercial formula. The hard red winter sample in test 2 was different from that in test 1. The judges' preference for odor in test 1 was not significant but their preference in taste was for soft red winter wheat. In test 2 there was a significant difference in their preference for both odor and taste, but in this test hard red spring was favored. Since test 1 was made at the time the machinery of baking, sampling, and judging was first set into motion, it was decided to use the wheat preference indicated in test 2 for the flour in the rest of the study. These results were probably more reliable. At the same time it was felt that while these samples of wheats were representative of the different classes, there might be as much difference among samples of the same classes of wheats obtained from different sources as among the different classes of wheats used for the experiment.

GRADE OF FLOUR. These flours were experimentally milled from hard red spring wheat and were unbleached. On the basis of taste in both tests, but for odor in only the first test, the judges made a significant choice in favor of the 85% patent flour.

BLEACHING. The flours were commercially milled 75% patent from hard red spring wheat. One sample was unbleached, the other two were treated with bleaches No. 1 and No. 2, respectively. There was no significance to the judgment on odor or taste in either test.

SPONGE AND STRAIGHT DOUGH. The flour used was commercially milled, bleached, 75% patent from hard red spring wheat. In test 1 there was no choice for odor while the preference for taste was on the borderline of significance in favor of the sponge method. In the second test the choice was significantly in favor of the sponge method for both odor and taste.

FERMENTATION AND BAKING TEMPERATURES. The flour was the same as in the previous test. The choice was not significant in test 1, but in test 2 the preference, as to both odor and taste, was significantly in favor of the higher fermentation temperature and the low baking temperature.

TYPE OF FORMULA. In both series of tests there was a significant choice in favor of the rich formula for both odor and taste. It must be borne in mind, however, that these tests were made on bread as an isolated food just before the lunch period and not upon bread at a meal.

FRESHNESS OF BAKED BREAD. In both tests 1 and 2, there was a preference for the odor and the taste of the 12-hour bread.

Discussion

In addition, separate analysis was made of the judgments of 16 individuals who had had experience in judging the quality of food products, including bread. The preferences of these experts corroborated those of the consumer group of 96. The smaller group, like the larger, preferred hard red spring, 85% patent flour, a rich formula, and 12-hour-old bread. They made no significant choice for fermentation and baking time, bleached or unbleached flour, or for method of mixing the dough.

Analyzing the judgments of the consumer group on paired or duplicate samples of each test, it was found that on the average they checked in their preference for the best sample in odor only 45% and in taste 43% of the time. The experts on the average checked their choice 47% of the time for odor and 40% for taste. Sixty-four percent of the consumer group duplicated their preference for odor in the "type of formula" test; 66% of the experts duplicated their choice for odor on the "type of dough" test. These were the best checks on preference.

Table II shows the distribution of ratings of those individuals who

TABLE II

NUMBER OF INDIVIDUALS DUPLICATING JUDGMENTS OUT OF 58 WHO JUDGED FRESHNESS AND 62 WHO JUDGED TYPE OF FORMULA

	Odor and taste	Odor	Taste
Freshness of bread	Number of judges	Number of judges	Number of judges
48 hours old	0	1	1
24 hours old	1	2	4
12 hours old	2	3	5
No choice	1	2	1
Total	4	8	11
Type of formula			
Lean	1	2	2
Medium	3	5	5
Rich	4	8	9
No choice	0	0	1
Total	8	15	17

judged freshness (58) and type of formula (62) in both tests and who duplicated their judgments for either odor or taste or both in all samples. This small group corroborated the preferences of the whole group, except in the odor of the 12-hour old bread, which fewer judges found desirable.

Analyzing the data to find which factor, odor or taste, had more influence when the judges ranked the bread "best," it was found that taste was the more important. It was fifteen times as important as odor in the wheat classes test, and ranged down to 1.5 times in the freshness test. It was four times as important as odor in the test on type of formula.

TABLE III

COMBINED SCORES FROM THE TWO TESTS ON FRESHNESS AND TYPE OF FORMULA, CALCULATED FROM JUDGES' RATINGS FOR QUALITY

	Freshness of bread					
	48 hours old		24 hours old		12 hours old	
Rating	Number rating	Score	Number rating	Score	Number rating	Score
Rating when smelled						
Excellent	14	56	19	76	31	124
Good	61	183	54	162	49	147
Fair	52	104	50	100	54	108
Poor	23	23	27	27	16	16
Total	150	366	150	365	150	395
Average	Fair +, 32.4		Fair +, 32.4		Good 2.6	
Rating when tasted						
Excellent	14	56	16	64	32	128
Good	52	156	67	201	47	141
Fair	59	118	43	86	51	102
Poor	22	22	21	21	17	17
Total	147	352	147	372	147	388
Average	Fair +, 32.4		Fair to good 2.5		Good 2.6	
	Type of formula					
	Lean		Medium		Rich	
Rating when smelled						
Excellent	9	36	21	84	44	176
Good	35	105	54	162	56	168
Fair	62	124	46	92	30	60
Poor	34	34	19	19	10	10
Total	140	299	140	357	140	414
Average	Fair +, 32.2		Good 2.6		Good 3.0	
Rating when tasted						
Excellent	7	28	19	76	40	160
Good	34	102	63	189	62	186
Fair	67	132	43	86	28	56
Poor	31	31	14	14	9	9
Total	139	293	139	365	139	411
Average	Fair 2.1		Good 2.6		Good 3.0	

The order of judging the bread samples had no significant effect on the judge's ratings, except in the first test on the type of formula.

Analyzing further the ratings given in the freshness and type of formula tests in which significant preferences were shown, it was found that 67% of the judges who duplicated their ratings for "best" odor in the freshness test, also gave the same rating for quality of the bread in the two tests. Eighty-two percent of this group duplicated their rating regarding the quality of the bread after tasting. In the type of formula test, 70% rated the quality of bread the same after smelling it and 83% after tasting it.

Table III shows the total scores for the breads obtained from the ratings given by all the judges in the freshness test assuming that the rating "excellent" equals 4 points, "good" 3, "fair" 2, "poor" 1. The 12-hour bread when smelled rated best in quality, but there seemed to be little difference in the quality of the 24-hour and 48-hour old bread. When tasted there was less superiority in the 12-hour old bread, but a greater difference between the 24- and 48-hour old bread. This relationship is different from that shown in Table I in that there is less significance to the preference for the odor of the 12-hour old bread than there is for the taste.

The quality of the bread improved in the opinion of the judges as the formula became richer, whether they tasted or smelled the bread.

Summary

In tests to show consumer preference as to taste and odor of bread made with different classes of wheat, grades of flour, bleaches, methods of mixing, fermentation and baking temperatures, types of formula, and freshness of the baked bread, a rich formula and fresh bread were preferred. Some significance may be attached to the grade of flour preferred, the choice being for 85% patent over straight and over 70% patent flour.

Taste was more important than odor in determining preference in flavor of bread.

Either the method used in this study is not sufficiently refined to detect small differences in taste or odor in bread, or the differences are unimportant.

Apparently the use of a large number of judges does not increase the validity of the results of the tests on bread flavor. From a small group of individuals who are accustomed to judging food quality the results were practically the same as with a larger untrained group.

The trained judges were no keener at discerning small differences in taste and odor of bread than the untrained group. This may be

due to the fact that the differences were below the threshold at which the effect of the factor under test is noticeable.

Further consideration should be given to the method employed in studying bread flavor.

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BAKING QUALITY OF FLOUR AS AFFECTED BY CERTAIN ENZYME ACTIONS. III. PURIFIED AMYLASE AND THE RELATIVE PROTEOLYTIC ACTIVITY OF AMYLOLYTIC AGENTS

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(Read at the Annual Meeting, June 1936)

Introduction

In two earlier papers (Read and Haas, 1934, 1936), we have directed attention to the beneficial influence exerted by certain proteolytically active agents on bread doughs characterized by varying degrees of "buckiness." Our studies have been further extended in the present paper and the relative proteolytic power of the saccharogenic agents tested has been determined by copper precipitation and by alcoholic titration. The importance of mellowing the gluten (within certain limits) by means of enzymic hydrolysis is receiving wider recognition and research workers in the United States and abroad have reported recent studies connected with some phase of the problem. The interesting paper by Lüers (1936) dealing with the changes in enzymes during malting reveals something of the complexity of the problems demanding attention and deserves thoughtful consideration. In relation to the baking industry, there is need for an intensive study of the basic principles involved.

According to the recent work of certain European investigators, such as Lüers and Rümmler (1933, 1935), and Chrzaszcz and Janicki

(1933a-d), more than two amylases are contained in barley malt. In addition to the saccharifying, liquefying, and dextrinizing functions, there exist certain specific substances (sisto-amylase) which inhibit amylolytic action, along with certain other specific substances (eleuto-amylase) which activate or increase the amylolytic power. Sisto-amylase paralyses the saccharifying, liquefying, and dextrinizing functions of amylase. It is reported to be most abundant in germinated buckwheat, especially in the roots and leaves of the germinated seed. The amount of sisto-amylase in barley malt was found sufficient to bind from 1/2 to 2/3 of the amylase. In wheat and rye malts approximately 1/4 of the amylase was said to be inactivated. The specific activating substances (eleuto-amylase) appear to be protein degredation products.

Concerning the proteases in malt, the researches of Lundin (1922), Lüers and Malsch (1929), R. H. Hopkins (1929, 1930), Von Euler, Myrbäck, and Myrbäck (1929), together with studies made by other workers, support the belief that malt contains a proteinase having a pH optimum of 5.0, and at least two peptidases which manifest optimum activity at pH 7.0 or slightly on the acid side.

Brief Review of Literature Regarding Certain Methods for Determining Protein Decomposition

Present methods for determining the amount of protein decomposition, resulting from either the normal fermentation of a bread dough or from that of the same dough reinforced by the addition of proteolytic agents, are quantitatively inadequate. Only an approximate evaluation of the degree of hydrolysis can be made by the best analytical procedures employing titration, precipitation, or liquefaction. Titration, involving alcohol or formaldehyde, measures only the increase in water soluble amino-nitrogen. The behavior of the gluten complex in the processes of baking may be materially influenced by lesser degrees of splitting. Protein precipitation methods, first developed by Ritthausen (1872), have been extensively employed to measure the non-protein nitrogen produced by proteolytic cleavage. Other workers have used gelatin liquefaction to estimate the degree of proteolysis, but this method, as commonly applied, permits results which appear to have only a qualitative value.

Protein Precipitation by Copper

Osborne and Leavenworth (1916) perfected the technic of precipitation with copper hydroxide for the preparation of such native proteins as edestin and gliadin. They found that complete precipitation required a certain excess of copper sulfate.

Bach and Oparin (1922) and Bach, Oparin, and Wähner (1927) adapted the principle of copper precipitation to their study of the formation of protease in germinating and in resting wheat seeds. They reported that wheat, when allowed to germinate for eight days, increased its protease activity about 30 times.

Blish (1918) developed certain refinements and applied the method to studies of proteolysis in wheat flour. He found that the removal of the true proteins from non-protein nitrogenous substances in water solution was practically complete.

Olsen and Bailey (1925) reported that the proper excess of copper sulfate was quite important, but difficult to adjust, and further improved the technic by using phenolphthalein to indicate the transition point.

Skovholt and Bailey (1935) studied the effect of mixing and fermentation upon the protein structure and colloidal properties of doughs. Copper hydroxide and tungstic acid were each used as separate protein precipitants. It is their opinion that hydrolytic or other chemical changes, not measurable by present analytical methods, may materially modify the gluten characteristics of the dough.

Titration Methods

TITRATION IN ALCOHOL.—Balls and Hale (1935), utilizing the principle of alcoholic titration devised by Foreman (1920), developed the following procedure for measuring the degree of proteolysis produced in a given flour by a 2-hour digestion period at 40° C.

A weight of 20 g. of fat-free flour was uniformly suspended in 100 cc. of water containing 3.0 cc. of 0.1 N hydrochloric acid, which buffered the system to a pH of approximately 5.0. At the beginning and at the close of the digestion period, 20 cc. of the flour suspension was centrifuged, and 10 cc. of the supernatant extract, which represents a 2-g. sample of the flour, was titrated to a distinct red with 0.1 N alcoholic potash in a 300 cc. Erlenmeyer flask, using 1 cc. of 1% 0-cresolphthalein as indicator. The addition of 20 cc. of 95% ethyl alcohol to the flask caused the red color to disappear, whereupon the titration with alkali was continued until the color reappeared. The final titration was made after the addition of 175 cc. of alcohol. As measured in terms of amino-nitrogen, their results indicate that proteolytic activity in normal flours is small.

In a later paper (Balls and Hale, 1936), these authors have published data to show the relative distribution of proteinase in different tissues of the wheat grain. Other data are also presented to direct attention to the decrease in proteolytic power which results from partial destruction (by oxidation) of the natural proteinase activator when flour is

bleached by chemical agents. Storage in air and the use of "bread improvers," such as potassium bromate, cause a similar impairment of the proteinase activating system. In their opinion, there is usually too much rather than too little proteinase in flour.

FORMOL TITRATION.—Utilizing Sörensen's formol titration, Cairns and Bailey (1928) found that sound high-grade flours showed only a small degree of proteolysis after subjection to a digestion (autolytic) period of 48 hours at 37° C. Brownlee and Bailey (1930) detected no measurable proteolysis in bread doughs after a 5-hour fermentation period. Herd (1931) reached the conclusion that the proteolytic activity of flour itself, as revealed by amino nitrogen, is of small value and without appreciable influence in a fermenting dough.

Samuels (1934) applied the method adopted by Brown (1923) for the formol titration of bacteriological media. Titrations were conducted in two stages, using phenol red as indicator and titrating to pH 8.0 with N/14 sodium hydroxide delivered from a micro-burette calibrated to 0.01 cc. The first titration measured the titratable acidity; the second the alkali required to neutralize the amino acids in solution and bring the formaldehyde to pH 8.0. Increments in titration became evident at pH 9.0. These were explained on the basis that they were not due to the production of amino acids in the usual sense of the term, but to groups intermediate between amino acids and proteases or peptones. Such groups have a low acid dissociation constant, and become titratable at pH 9.0.

Blagoveschenski and Yurgenson (1935) reported data to support their conclusion that flour contains certain enzymic factors which may disaggregate the gluten complex without producing free amino-nitrogen. The elaborate technic employed in this investigation imposed a considerable number of variables. Their experimental work entailed the separation of the flour proteins into four fractions represented by (1) leucosin and non-protein nitrogen; (2) gliadin; (3) globulin; (4) and glutenin; and a study of these separate fractions subjected to the action of proteases extracted from bread flour and also those extracted from yeast. The maximum activity of the protease which they extracted from flour occurred at pH 8.5, but did not produce a true proteolysis. The proteases of yeast showed maximum activity in the pH range 4.8 to 6.5, produced a true proteolysis, but exhibited no proteolytic activity at pH 8.5.

Samuels (1935) reported that the amino acid content of an unyeasted flour dough increased slowly with time. In a yeasted flour dough the amino acid production progressed about twice as rapidly, but only for about one hour, at which time the content decreased to an almost constant value, because of the rate of utilization of amino

nitrogen by the yeast. These results fail to confirm those of Blagoveschenski and Yurgenson (cited above).

Samuels has suggested that the discrepancies may be partly explained by differences in technic, *e.g.*, they allowed the enzymes extracted from 0.25 g. of flour or yeast to act on the gluten from about 10 g. of flour, whereas he allowed the enzymes to act *in situ*. The flour or yeast enzymes were probably not fully extracted by Blagoveschenski and Yurgenson, and the increase in amino nitrogen reported in their control solutions may be accounted for by the action of the unextracted portion which remained bound to the gluten.

Gelatin Liquefaction

In two recent papers dealing with the nature of the action of potassium bromate and other similar agents, Jørgensen (1935) has presented data from which he concludes that the advantages obtained by the use of certain "gluten developers" are to be attributed to their inhibitory action on the proteinases of wheat flour, with the net result that the gas retaining capacity of the gluten film is thereby increased.

Relative proteolytic activity was measured on flour suspensions and also on gelatin, the latter according to the technic employed by Stockham (1920), wherein the number of hours required to effect complete liquefaction is taken as a measure of proteolytic power. Other investigators have made similar use of gelatin liquefaction to measure relative proteolytic activity.

To determine soluble nitrogen, 25 g. of flour were extracted for 2 hours at 35° C. with 75 cc. of water and 25 cc. of phosphate buffer (pH 5.8) plus certain quantities of the oxidizing and proteolytic factors. The amount of the "gluten developing" salts in the reaction mixtures was chosen so that the resulting concentration was always 0.003 molar. The depression exerted by the salt on proteolytic activity was expressed in terms of percentage of soluble nitrogen.

In a more recent paper, Jørgensen (1936) has described other similar experimental tests to demonstrate that flour itself has a notably high content of proteinase, but no severe hydrolysis of the gluten is manifested until the proteinase has been activated by the presence of some substance such as glutathione. The HS-group in the molecule of glutathione is believed to be the responsible factor.

That yeast is a good source of glutathione was discovered by F. G. Hopkins (1921), who also (1929) found it to be a tripeptide, derived from glycine, glutamic acid, and cysteine. On the basis of certain tests which Jørgensen conducted with a concentrated water extract of yeast (boiled 10 minutes and filtered clear), he concluded that flour proteinase is definitely activated by this constituent of yeast during

dough fermentation, and accepts this as additional evidence in support of his contention regarding the nature of the action of potassium bromate.

Contrary to the opinions advanced by Jørgensen, Hesse (1935) holds to the generally accepted view that the proteolytic activity of normal bread flour is too weak to materially influence the gluten during fermentation and baking. Other influences, such as changes in pH, appear to be of greater importance.

Experimental

Preparation of Enzyme Material

Amylase Preparations.—In the course of our studies, it was considered advisable to conduct baking tests with purified amylase. The method described by Sherman, Caldwell, and Doebbeling (1934) was selected. It involves successive fractional precipitation with ammonium sulfate; resolution and dialysis of the most active fractions to remove the sulfate; a fractional precipitation by alcohol; resolution of the most active alcohol precipitate, which is then used to obtain the final precipitate with alcohol plus ether. It is essential that all solutions and preparations be kept at a low temperature.

Our preparations were twice carried through the prescribed scheme of sulfate precipitation, with subsequent dialysis. The last two steps in purification were effected by first fractionating with alcohol, followed by a final precipitation with the alcohol-ether mixture. The more active amylase preparations obtained by us showed a saccharifying power varying from 15 to 20 times that of the original malt flours used to produce them. The purified fractions were kept either in solution or in suspension and all experimental tests with them were conducted before they showed any deterioration. Their saccharifying power was determined on the basis of dry matter contained therein.

We found, however, that our amylase preparations were also highly proteolytic. The proteinase accompanied the amylase in the process of purification and, apparently, was purified to a corresponding degree. We had hoped that this method would yield a product having little or no proteolytic activity. To accomplish such an end, it appears that one must start with an amylolytic agent containing no protease.

Preparation of Malt Flours.—Barley malt flour No. 5 was prepared from barley which had been allowed to germinate for seven days at near 55° F. The average length of the acrospire was approximately 16 mm. The amylase and protease content of this flour was

relatively much higher than is true for the average run of malt flours. It yielded a saccharogenic index of 520 on flour No. 133375. Barley malt flour No. 4 was prepared from green barley malt supplied by A. Schwill and Company. It gave an index of 170.

AQUEOUS EXTRACTS OF ENZYMIC AGENTS.—With the exception of Merck's diastase of malt (extracted with 5 times its weight of water) the aqueous extracts were prepared by mixing each malt product with 3 times its weight of cold distilled water and allowing to stand, with frequent stirring, for two hours in refrigerator. Most of the solid material was removed by centrifuging at high speed. The supernatant liquid was then further clarified by use of the super centrifuge before being subjected to fractional precipitation with ammonium sulfate.

Procedures Used to Determine Proteolysis

COPPER-PROTEIN PRECIPITATION.—Determinations were made on 2 g. of flour digested with small amounts of the active agent for 48 hours at 35° C. From time to time a few drops of toluene were added to prevent bacterial decomposition. The protein fraction was precipitated in accordance with the technic adopted by Bach and his coworkers (*loc. cit.*) and the refinements devised by Blish and by Olsen and Bailey.

TABLE I

SACCHAROGENIC INDICES [1]

(*All determinations made on flour No. 133375.*)

Saccharogenic product	Purified precipitates			
		Alcohol-ether precipitate from second 20 to 35 g. sulfate fraction	Second 20 to 35 g. sulfate fraction after dialysis	Second 10 to 20 g. sulfate fraction after dialysis
	Index values			
Malt diastase Merck's (USP–IX)	455	7,160	5,428	2,820
Barley malt flour No. 4	170	—	3,376	1,930
Barley malt flour No. 5	520	—	6,236	3,152

[1] The term "saccharogenic index" as employed by us denotes the number of milligrams of anhydrous maltose capable of being produced by 100 mg. of the active agent from 10 g. of the flour sample during the course of one hour at 30° C. Obviously, the index value must exclude the autolytic activity of the flour. The customary procedure is to determine the maltose produced by 5 g. of the flour when digested with 20 mg. of the saccharogenic agent and then calculate the value for 100 mg. on 10 g. of flour. It is recognized that the index figure is influenced by the quantity of enzymic preparation which is allowed to react. Small dosages tend to give relatively higher index values.

Titrations in alcohol were made by applying the method of Balls and Hale as developed by these investigators for the purpose of measuring proteolytic activity in flours. The technic involved has already been described.

The attempts to secure highly purified amylase, essentially free from proteinase, did not meet with the success desired. Proteinase accompanied the amylase through the various steps of purification, and was apparently purified to an equal degree.

The relative saccharogenic power of several of the purified amylase fractions and the enzymic agents from which they were derived are shown in Table I. The relative proteolytic power of these preparations, as determined by copper precipitation, is presented in Table II,

TABLE II

RELATIVE PROTEOLYTIC POWER OF AMYLOLYTIC AGENTS

By Copper Precipitation Method—Flour No. 133375
(Two grams flour digested 48 hrs. at 35° C.)

Enzymic agent	Flour (Autolytic)	Diastase of malt (USP–IX)	Taka-diastase	Wheat malt H	Barley malt No. 4	Barley malt No. 5
Saccharogenic index		455	500	475	170	520
Amount added, mg.	None	20	20	20	20	20
Non-precipitated nitrogen, %	11.5	66.4	68.1	16.0	12.9	19.6
Non-precipitated nitrogen, mg.	5.2	30.0	31.0	7.3	5.9	8.4

Purified amylase from diastase of malt (USP–IX)
(Second series of sulfate fractionations)

Enzymic agent	Alcohol-ether fraction	65% alcohol fraction	Second 10 to 20 g. fraction	Second 20 to 35 g. fraction
Amount added, mg.	2	2.2	2	2
Non-precipitated nitrogen, %	69	83	67.4	66
Non-precipitated nitrogen, mg.	31.2	37.9	30.5	30.2
Non-precipitated nitrogen per milligram dosage	15.6	17.2	15.2	15.1

Purified amylase fractions from malt flour No. 4
(Second series of sulfate fractionations)

Amount added, mg.	4	6	4	
Non-precipitated nitrogen, %	17	30.6	15.5	
Non-precipitated nitrogen, mg.	7.7	13.9	7.0	
Non-precipitated nitrogen per milligram dosage	1.9	2.3	1.8	

while Table III records the relative proteolytic activity as evidenced by titration in alcohol. Baking data are shown in Table IV.

TABLE III

RELATIVE PROTEOLYTIC ACTIVITY BY TITRATION

Enzymic agent	Cc.N/10 KOH [1]
Control (autolytic)	0.40
Barley malt No. 5	0.70
Malt diastase (USP–IX)	1.20
Taka diastase	1.50
Papain	3.30
Pepsin	1.60

[1] Ten grams of flour digested with 100 mg. (1%) of enzymic agent for 2 hours at 40° C. Values given in Cc. N/10 KOH represent 2 g. of flour, and record the excess titration over non-autolytic control. Flour No. 133375 was used for all determinations.

DISCUSSION

A study of the literature reveals a wide divergence of opinion concerning the part played by proteolysis in bread doughs during fermentation and baking. Certain investigators, such as Jørgensen, believe that proteolysis proceeds to such a degree as to result in injury to the gluten complex.

TABLE IV

RESULTS OF BAKING TESTS WITH SPONGE DOUGHS [1]

Enzymic agent	Amount added	Loaf volume	Texture	Grain	Ovenspring
	Grams	*Cc.*	*Score*	*Score*	
Flour No. 133375—Saccharogenic activity 260					
Control	None	2067	87	87	Poor
Malt diastase (USP–IX)	0.350	2510	100	100	Good
Taka diastase	0.300	2520	100	100	Good
Barley malt No. 4	3.500	2205	95	94	Poor
Amylase Preparations from Barley Malt Flour No. 4					
Flour No. 133375—Saccharogenic activity 260					
(From second series of sulfate fractions unless otherwise indicated)					
65% alcohol fraction	0.20	2310	93	93	Fair
20–35 g. fraction	0.48	2457	99	97	Fairly good
20–35 g. fraction	0.60	2500	99	97	Fairly good
10–20 g. fraction (first series)	1.20	2425	96	94	Fairly good
Safranine-protease precipitate from 0.60 g. of 20–35 g. fraction	—	2450	98	96	Fairly good
Safranine-protease precipitate from 0.260 g. of 65% alcohol fraction	—	2400	100	99	Fairly good

[1] Scaling weight 500 g. Enzyme preparations added to sponge.

TABLE IV—*Continued*

Amylase Preparations from Diastase of Malt (U.S.P.–IX)
Flour No. 133375—Saccharogenic activity 260
(From second series of sulfate fractions)

Enzymic agent	Amount added	Loaf volume	Texture	Grain	Ovenspring
	Grams	*Cc.*	*Score*	*Score*	
Alcohol-ether fraction	0.011	2345	97	97	Fair
20–35 g. fraction	0.012	2478	100	100	Good
20–35 g. fraction	0.024	2600	100	100	Good
20–35 g. fraction	0.100	2680	100	100	Good
20–35 g. fraction	0.150	2712	100	100	Good
Safranine-protease precipitate from 0.1 g. of 20–35 g. fraction	—	2665	100	100	Good

Flour No. 142248—Saccharogenic activity 203

Control	None	1902	81	81	Poor
Malt diastase (USP–IX)	0.350	2522	100	100	Good
Barley malt No. 5	3.500	2072	95	95	Poor

Amylase Preparations from Diastase of Malt (U.S.P.–IX)
Flour No. 142248—Saccharogenic activity 203
(From second series of sulfate fractions)

20–35 g. fraction	0.020	2534	100	100	Good
20–35 g. fraction	0.030	2569	100	100	Good
Safranine-protease precipitate from 0.050 g. of 20–35 g. fraction	—	2507	100	100	Good
Filtrate from 0.050 g. of 20–35 g. fraction after protease precipitate	—	2219	96	94	Fair

Amylase Preparations from Barley Malt Flour No. 5
Flour No. 142248—Saccharogenic activity 203
(From second series of sulfate fractions)

20–35 g. fraction	0.280	2206	98	98	Fair
20–35 g. fraction	0.600	2490	100	99	Good
Safranine-protease precipitation from 0.600 g. of 20–35 g. fraction	—	2435	99	99	Good
Filtrate from 0.600 g. of 20–35 g. fraction after protease precipitation	—	2124	92	92	Poor

Figure 1. Baking results with flour No. 133375, showing effect on crumb structure and loaf volume produced by small amounts of fractional precipitates derived from an extract of barley malt flour No. 4 (Extract II). Additions were made to the sponge.

Figure 2. Baking results with flour No. 133375, showing effects on crumb structure and loaf volume produced by small amounts of fractional precipitates derived from an extract of barley malt flour No. 4 (Extract III), also from an extract of Merck's diastase of malt (USP-IX). Precipitates were added to the sponge.

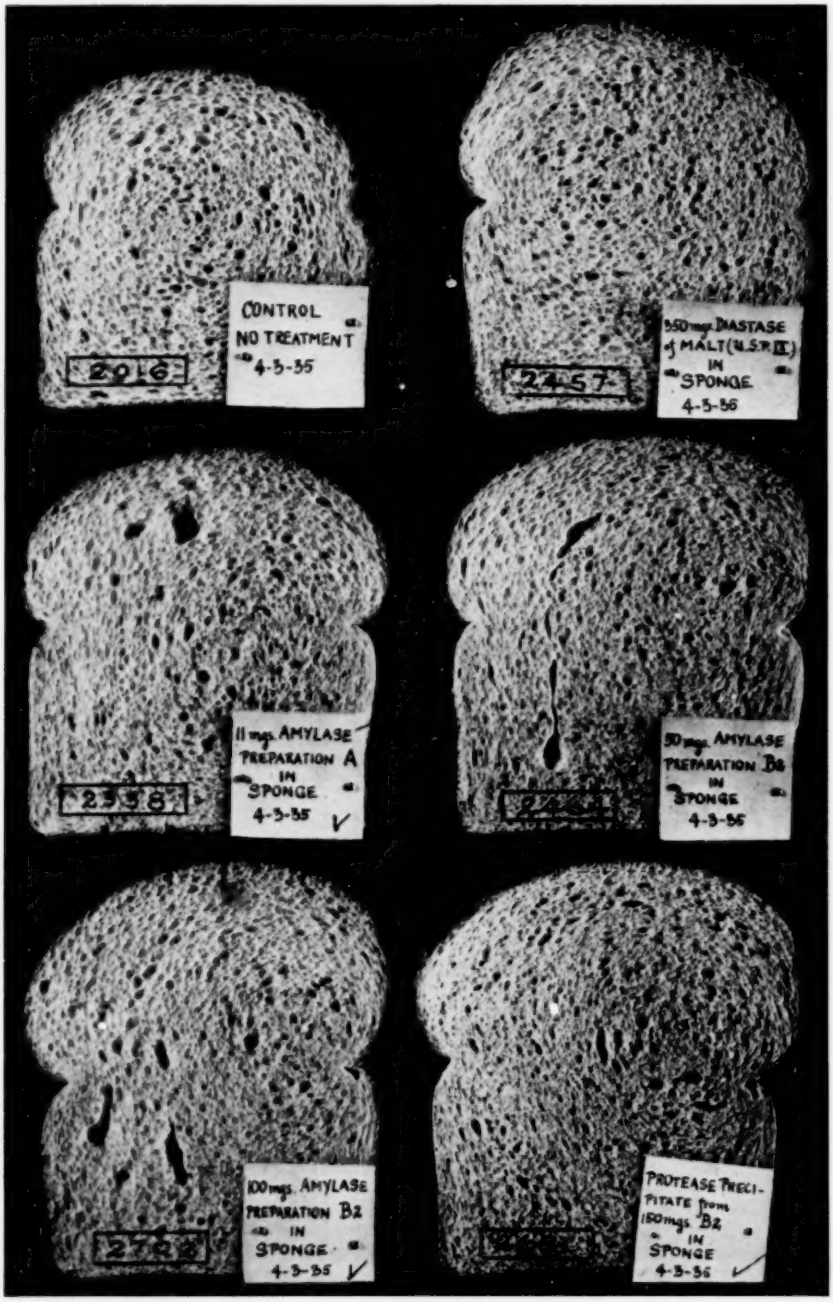

Figure 3. Baking results with flour No. 133375, showing effects on crumb structure and loaf volume produced by small amounts of fractional precipitates derived from an extract of Merck's diastase of malt (USP–IX). Preparation B_2(20–35 g. fraction after dialysis). Preparation A (65% alcohol precipitate derived from 20–35 g. fraction). Additions made to sponge.

If the conclusions reached by Jørgensen are tenable, *viz.*, that the beneficial effects derived from the use of bromate result from its inhibitory action on proteolysis, it would follow that the proteinase activity is of sufficient magnitude to injure the gluten in flours which appear to be benefited by the addition of bromate. It is generally accepted that the proteolytic power of wheat flour milled from sound grain is insufficient to cause any appreciable hydrolysis of the gluten. It appears, however, that normal bread flour does have a relatively high content of proteinase, which may readily be set to work by the presence of some activating agent such as glutathione or cysteine hydrochloride. The gluten may even be seriously damaged by an overdosage of such an activator. On the contrary, the baking quality of many bread flours is greatly improved by suitable additions of cysteine hydrochloride, glutathione, or of certain agents with a high content of protease.

Many fundamental questions arise which demand further investigation before they can be sufficiently clarified to be given general acceptance. Various problems concerned with gluten quality are of long standing and still await solution. The complexity of their nature provides a fertile field for research.

The baking test data which we have presented in Table I show the benefits to be derived by stepping up proteolytic activity in doughs which are commonly called "bucky." Many flours give rise to doughs having glutens which show varying degrees of "buckiness."

Doughs of this character are materially improved in baking quality by additions of suitable quantities of protease, or of some activator for protease such as cysteine hydrochloride, or glutathione. We have used the former with excellent results. Obviously, it is necessary not to overstimulate the proteinase activity of the flour. It may be added also that a number of other flours which did not give rise to doughs of a "bucky" character showed no ill effects from smaller increments of proteinase. Most of them, in fact, were appreciably benefited. It would appear that the benefits arise chiefly from a certain degree of gluten hydrolysis, but this does not exclude other advantages which may result indirectly.

Summary

Numerous baking tests have been made, using small amounts of purified amylase. The fractional precipitation method of purification failed to remove proteinase. In fact, the proteolytic power appeared to be intensified to the same extent as was the amylolytic. Baking test results are recorded in Table IV and illustrated in Figures 1, 2, and 3. The marked improvements in baking quality are attributed

primarily to proteolytic activity. The same advantages may be gained by using equivalent quantities of proteinase which has been separated (by precipitation with safranine) from its accompanying amylase.

Excessive dosages of proteinase or of some proteinase activator, such as glutathione or cysteine hydrochloride, will ruin the baking properties of any gluten. A stiff dough may readily be reduced to the consistency of a thick soup. This would indicate that flour itself is adequately supplied with proteinase, but it is not present in a very active state. As a consequence, flours which give rise to "bucky" doughs may be greatly improved in baking quality by suitable increments of proteinase.

The relative saccharogenic power of the purified preparations is given in Table I. Proteolytic activity, as evidenced by copper precipitation, is presented in Table II, and Table III contains similar data determined by titration in alcohol.

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A COLLABORATIVE STUDY OF THE BLISH-SANDSTEDT, SCHOORL, AND BERTRAND METHODS FOR DETERMINING REDUCING SUGARS IN FLOUR DIASTATIC ACTIVITY MEASUREMENTS

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(Read at the Annual Meeting, June 1936)

In the measurement of the diastatic activity of flour, the Rumsey method of diastasis, with certain modifications such as pH control, has found general acceptance and is widely used. Different methods are used in different laboratories for determining the quantities of reducing sugars in the flour extracts present after diastasis, among which may be mentioned the Blish-Sandstedt, Schoorl, and Bertrand methods. All of these methods are volumetric procedures. In the Blish-Sandstedt procedure, standard potassium ferricyanide is employed and the unreduced ferricyanide is titrated against standard sodium thiosulphate. The Schoorl and Bertrand procedures are copper reduction procedures; in the former the unreduced copper is titrated against standard sodium thiosulphate solution while in the latter method the reduced copper is filtered off, dissolved in ferric sulphate solution and titrated with standard potassium permanganate solution.

Methods Used

The Blish-Sandstedt method.—Cereal Chemistry **10:** 189–202; A. A. C. C. Cereal Laboratory Methods, pp. 56–58 (1935); Cereal Chemistry **12:** 494–504.

The Schoorl method.—The flour was digested as in the above procedure, and 25 cc. of the clear filtered extract was analyzed as described in Cereal Chemistry **10:** 495; or A. A. C. C. Cereal Laboratory Methods, p. 18 (1935). Milligrams of maltose per 10 g. of flour was obtained by multiplying by 4 the number of milligrams of monohydrate maltose as found in the table in the above mentioned publications.

The Bertrand method.[1, 2]—The flour was digested as in the above procedures and 10 cc. of the clear filtered extract transferred to a 250 cc. Erlenmeyer flask, 20 cc. of water added, then 10 cc. each of solutions A and B (made up as described in the Schoorl Method) bringing the total up to 50 cc. The liquid was brought to a boil in 3 minutes, boiled for exactly 2 minutes, and then cooled and filtered through a Bertrand filter using asbestos prepared for sugar determinations. The residue was washed several times with water at 60° C. and was then dissolved in 10 cc. of ferric sulphate solution (50 g. ferric sulphate, 200 cc. concentrated H_2SO_4,

[1] Bertrand, G. Le dosage des sucres reducteurs. Bull. soc. chim. Paris **35:** 1285 (1906).

[2] Mathews, Albert P. Physiological Chemistry, Fifth Edition (1930), William Wood & Co., New York (p. 952).

diluted to 1 liter). The filtrate and asbestos filter were then transferred to a flask with enough 60° C. water to make about 100 cc. volume and titrated with .05 N $KMnO_4$. Milligrams of maltose (monohydrate) per 10 g. of flour corresponding to the titration value found in Table I.

TABLE I

BERTRAND TABLE FOR DIASTATIC ACTIVITY

.05 N $KMnO_4$	Maltose per 10 g. flour (Mono hydrate)	.05 N $KMnO_4$	Maltose per 10 g. flour (Mono hydrate)
Cc.	*Mgs.*	*Cc.*	*Mgs.*
4.0	109	8.6	245
4.1	113	8.7	248
4.2	115	8.8	252
4.3	118	8.9	255
4.4	121	9.0	258
4.5	124	9.1	261
4.6	127	9.2	264
4.7	131	9.3	266
4.8	133	9.4	269
4.9	136	9.5	273
5.0	139	9.6	276
5.1	141	9.7	279
5.2	144	9.8	282
5.3	147	9.9	284
5.4	151	10.0	287
5.5	154	10.1	291
5.6	157	10.2	294
5.7	160	10.3	297
5.8	163	10.4	300
5.9	166	10.5	302
6.0	169	10.6	305
6.1	173	10.7	308
6.2	176	10.8	312
6.3	178	10.9	314
6.4	181	11.0	317
6.5	184	11.1	319
6.6	187	11.2	322
6.7	189	11.3	325
6.8	193	11.4	328
6.9	196	11.5	332
7.0	198	11.6	335
7.1	201	11.7	338
7.2	204	11.8	341
7.3	207	11.9	344
7.4	211	12.0	346
7.5	213	12.1	349
7.6	216	12.2	353
7.7	218	12.3	356
7.8	221	12.4	358
7.9	224	12.5	361
8.0	227	12.6	363
8.1	231	12.7	366
8.2	234	12.8	369
8.3	237	12.9	373
8.4	239	13.0	378
8.5	242		

Experimental

The Methods of Analysis Committee undertook a collaborative study of these three procedures for the purpose of obtaining a comparison of the units of measurement and the relative variability within and between the methods in different laboratories on flour samples of different diastatic value.

Subsamples were submitted to collaborators from flour samples having the following analysis:

Sample number	Moisture	Ash	Protein	Origin
	%	%	%	
1	12.8	0.40	10.65	Southwest
2	10.3	.39	14.60	Northwest
3	10.2	.40	14.50	Northwest
4	13.7	.40	12.00	Southwest

The first sample had been submitted to the Pioneer Section as the October check sample. Twenty chemists, all of whom used the Blish-Sandstedt procedure, reported an average diastatic value of 171.4 mg. maltose per 10 g. flour (S.E. 6.28 mg.). All of the flour samples used in the study carried their natural malt value except flour No. 4 which was treated with 0.25% finely ground malted wheat flour. This was thoroughly mixed into the flour with a mechanical agitator.

Sample No. 5, the maltose used in this study, was taken from a lot of maltose suguar supplied by the Pfanstiehl Chemical Company, Waukegan, Illinois, and was labeled "C.P. Maltose, Hydrate, Sp. R. + 131°, Moisture 0.1%, Ash 0.1%." Analysis, however, of one subsample showed a 0.7% moisture loss in a vacuum desiccator over H_2SO_4 in 16 hours at room temperature, and 5.7% loss in a vacuum oven overnight at 95° C. In the analysis of this sugar, each chemist was given the following instructions:

> Make up a 200 cc. solution so that it will contain 2.8 g. of the sugar. To 108 cc. of buffer solution add 30 cc. of the sugar solution, then add 6 cc. of 10% H_2SO_4 and 6 cc. of 12% tungstate solution. Test the solution as you would a flour extract and report as equivalent to milligrams of maltose per 10 g. of flour. When using 30 cc. of the sugar solution this should equal 280 mg. maltose per 10 g. of flour for a theoretically 100% pure monohydrate maltose sugar.

Each collaborator in these studies sent a sample of the buffer solution used in the flour digestion to the committee for pH determination. Dr. Earl Working, Kansas State College, determined the pH values and reported that the solutions of all but two collaborators were almost within experimental error. Collaborators and pH values reported were

as follows: (A) W. F. Geddes, 4.65; (B) Quick Landis, 4.75; (C) M. J. Blish, 4.63; (D) F. C. Hildebrand, 4.58; (E) C. E. Mangels, 4.65; (F) J. T. Flohil, 4.67; (G) Betty Sullivan, 4.65, and (H) C. F. Davis, 4.65.

TABLE II

DATA FROM COLLABORATORS COMPARING THREE METHODS FOR DETERMINING MALTOSE SUGAR IN FLOUR EXTRACTS

(Values expressed as milligrams maltose per 10 g. flour)

Collaborator	Blish-Sandstedt method				Schoorl method				Bertrand method				All methods
Sample No. 1													
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	Mean
A	167	168	167	167.3	167	165	170	167.3	169	172	171	170.6	168.4
B	166	168	168	167.3	162	160	161	161.0	151	154	158	154.3	160.9
C	170	168	170	169.3	164	161	164	163.0	173	173	173	173.0	168.4
D	173	173	173	173.0	160	162	160	160.6	168	168	171	169.0	167.5
E	166	166	166	166.0	171	171	171	171.0	175	172	169	172.0	169.7
F	160	160	160	160.0	160	160	160	160.0	161	158	165	161.3	160.4
G	172	172	172	172.0	172	172	177	176.3	166	169	173	169.3	171.7
H	168	168	168	168.0	167	167	166	166.3	155	158	155	156.0	163.5
Mean, all collaborators				167.9				165.4				165.7	166.3
Sample No. 2													
A	202	202	205	203.0	213	210	210	211.0	202	202	201	201.6	205.2
B	204	204	202	203.3	205	205	206	205.3	198	197	200	198.3	202.3
C	202	204	199	201.6	197	197	199	197.6	212	213	211	212.0	203.8
D	220	220	220	220.0	202	202	204	202.6	210	210	212	210.6	211.1
E	205	205	205	205.0	211	211	211	211.0	216	215	213	214.6	210.2
F	220	218	218	218.6	209	211	211	209.6	198	208	198	201.3	210.1
G	214	214	214	214.0	214	220	223	219.0	210	213	213	212.0	215.0
H	215	215	213	214.6	211	211	214	212.0	204	204	207	205.0	210.4
Mean, all collaborators				210.0				208.6				206.9	208.5
Sample No. 4													
A	289	290	290	289.6	310	313	315	312.6	302	300	302	301.3	301.2
B	292	292	294	292.6	312	310	312	311.3	297	296	299	297.3	300.4
C	310	315	315	313.3	316	311	316	314.3	317	318	319	318.0	315.2
D	321	321	321	321.0	308	310	314	310.6	319	319	317	318.3	316.7
E	301	301	301	301.0	332	332	332	332.0	322	323	320	321.6	318.2
F	314	318	314	315.0	314	314	314	314.0	312	312	318	314.0	314.4
G	312	318	318	316.0	323	323	323	323.0	312	314	312	312.6	317.2
H	322	322	322	322.0	328	327	328	327.6	311	313	310	311.6	320.3
Mean, all collaborators				308.8				318.2				311.8	312.9

TABLE II—*Continued*

Collaborator	Blish-Sandstedt method				Schoorl method				Bertrand method				All methods
Sample No. 3													
A	278	276	276	276.6	305	305	304	304.6	294	291	292	292.3	291.2
B	276	272	274	274.0	287	286	287	286.6	273	277	278	276.0	278.9
C	290	292	291	291.0	293	297	292	294.0	297	294	300	297.0	294.0
D	295	294	295	294.6	280	284	282	282.0	279	282	279	280.0	285.5
E	296	298	296	296.6	315	317	315	315.6	309	308	308	308.3	306.9
F	292	292	296	293.3	309	304	306	306.3	305	298	307	303.3	301.0
G	300	300	296	298.6	308	303	307	306.0	300	300	300	300.0	301.5
H	300	300	302	301.3	309	308	309	308.6	298	295	299	297.3	302.2
Mean, all collaborators				290.7				300.5				294.3	295.1

All flours

Collaborator	A	B	C	D	E	F	G	H	All
Blish-Sandstedt method	234.2	234.3	243.8	252.2	242.2	246.8	250.2	251.2	244.3
Schoorl method	248.9	241.1	242.2	239.0	257.4	247.7	255.4	253.7	248.2
Bertrand method	241.5	231.5	250.0	244.5	254.2	245.0	248.5	242.4	244.7
All methods	241.5	235.6	245.3	245.2	251.2	246.5	251.4	249.1	245.75

Discussion of Results

The experimental results obtained by all collaborators with the four flours are listed and averaged in Table II. Differences between collaborators due to variability in the mean values for all flours and all methods give a range of 235.6 units for collaborator B to 251.4 units for collaborator G. The variation between methods is due to variability in the mean values for all flours and all collaborators for each of the three methods, namely: 244.3, 248.2, and 244.7 for the Blish-Sandstedt, Schoorl, and Bertrand methods respectively. The Blish-Sandstedt and Bertrand methods give similar results, while the Schoorl method gives definitely high values. Collaborators did not secure the same relative results for the three methods. The collaborators fall in order of increasing values as follows: Blish-Sandstedt procedure—A, B, E, C, F, G, H, D; Schoorl—D, B, C, F, A, H, G, E; Bertrand—B, A, H, D, F, G, C, E.

The three methods placed the flours in the same order, but the range of values differ. For the Blish-Sandstedt method the mean values (for all collaborators) ranged from 167.9 to 308.9 or 141 units; for the Schoorl method—165.4 to 318.2 or 152.8 units; for the Bertrand method —from 165.7 to 311.8 or 146.1 units. From Table III and Figure 1 it

TABLE III

SUMMARY OF DATA FROM ALL COLLABORATORS AND ANALYSIS OF VARIANCE FOR INDIVIDUAL FLOURS

(Values expressed in milligrams maltose per 10 g. flour)

Sample No.	Mean all methods	Mean	Difference from all methods, mean	Standard error between collaborators	Standard error within triplicates
		Blish-Sandstedt method			
1	166.3	167.9	+1.6	3.99	.61
2	208.5	210.0	+1.5	7.54	1.41
4	295.1	290.7	−4.4	9.98	1.58
3	312.9	308.9	−4.0	12.67	1.86
Mean all samples		244.4		8.54	1.36
		Schoorl method			
1	166.3	165.4	− .9	5.09	1.59
2	208.5	208.6	+ .1	6.54	1.97
4	295.1	300.5	+5.4	11.67	1.82
3	312.9	318.2	+5.3	8.25	1.80
Mean all samples		248.2		7.89	1.79
		Bertrand method			
1	166.3	165.7	− .6	7.40	2.60
2	208.5	206.9	−1.6	6.49	2.41
4	295.1	294.3	− .8	11.15	1.88
3	312.9	311.8	−1.1	8.47	1.73
Mean all samples		244.7		8.38	2.15
		Mean standard error all methods			
					All collaborators
1				5.49	1.60
2				6.86	1.93
4				10.93	1.76
3				9.80	1.80

can be seen that the three methods give very close agreement on samples 1 and 2, but for high values the Schoorl method gives definitely higher values. This was supported by the data from collaborator H (Table V and Figure 1) testing maltose sugar (sample No. 5) in solutions of different concentration by the three methods. Collaborator H reported that in these results, the Bertrand values would be slightly higher if the work were repeated and a more definite end-point taken in the titrations.

Collaborator A, reporting the results of reducing sugar tests by the Quisumbing-Thomas [3] gravimetric method on aliquots from the extracts

[3] Association of Official Agricultural Chemists. Official and Tentative Methods of Analysis, Third Edition (1930), pp. 112–113.

tested by each of the three methods studied in this report, found for samples 1, 2, 3, 4, and 5; 165.3, 212.8, 313.6, 305.9, and 261.0 mg. maltose per 10 g. of flour respectively. These results compare closely with the results obtained for the Schoorl method.

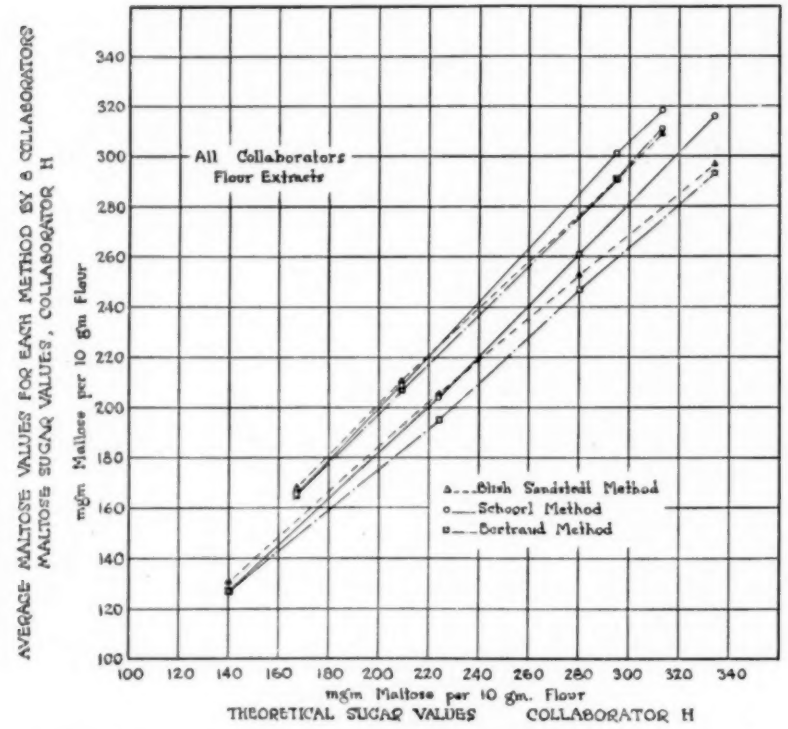
AVERAGE MALTOSE VALUES FOR EACH METHOD BY 8 COLLABORATORS
MALTOSE SUGAR VALUES, COLLABORATOR H
All Collaborators
Flour Extracts
mgm Maltose per 10 gm Flour
Blish Sandstedt Method
Schoorl Method
Bertraud Method
100 120 140 160 180 200 220 240 260 280 300 320 340
mgm Maltose per 10 gm. Flour
THEORETICAL SUGAR VALUES COLLABORATOR H

Fig. 1. Average maltose values of three methods, 8 collaborators. Upper curves refer to collaborative results on flour extracts. Average value of each method plotted against average of all methods. (Table III.) Lower curves refer to results of collaborator H using sugar solutions. Actual sugar values plotted against theoretical values for a 100% pure maltose sugar. (Table V.)

The data for each sample was submitted to a separate variance analysis and the results are summarized in Table III. Considering the mean standard error between collaborators on all samples, we find the lowest value in the instance of the Schoorl method, the Bertrand and Blish-Sandstedt values are close together with the highest for the Blish-Sandstedt. Considering the variance within triplicate tests or experimental error, the Blish-Sandstedt method gives the lowest values and the Bertrand the highest.

The higher experimental error for the Bertrand and Schoorl methods was possibly due in part to the difficulty in boiling each Fehling solu-

TABLE IV

SAMPLE NO. 5, MALTOSE SUGAR, THEORETICAL MALTOSE VALUE EQUAL TO 280 MGMS.

(Maltose expressed in flour values)

Collaborator	Blish-Sandstedt method				Schoorl method				Bertrand method				All Methods
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	Mean
A	257	255	255	255.6	275	274	276	275.0	258	258	261	259.0	263.2
B	240	242	244	242.0	249	248	247	248.0	245	243	246	244.6	244.9
C	251	253	252	252.0	255	255	252	254.0	258	264	258	260.0	255.3
D	262	262	262	262.0	258	257	257	257.3	261	261	258	260.0	259.8
E	252	252	252	252.0	266	266	266	266.0	253	253	253	253.0	257.0
F	250	250	252	250.6	256	254	265	258.3	257	256	257	256.6	255.2
G	260	260	260	260.0	265	261	261	262.3	258	261	261	260.0	260.8
H	255	253	251	253.0	261	261	261	261.0	245	251	245	247.0	253.6
Mean, all collaborators				253.4				260.2				255.0	256.2
Difference from mean of all methods				−2.8				+4.0				−1.2	
Percent of theoretical				90.5				92.9				91.08	
Standard error between collaborators				6.14				8.07				6.19	
Standard error within triplicates				1.15				2.37				2.23	

TABLE V

COMPARATIVE TESTS OF PURITY OF A MALTOSE SUGAR SAMPLE OBTAINED BY COLLABORATOR H USING THREE FLOUR TESTING METHODS AT DIFFERENT SUGAR CONCENTRATIONS

(Maltose value expressed as milligrams maltose per 10 g. flour)

Theoretical maltose value	Blish-Sandstedt method			Schoorl method			Bertrand method		
	Milligrams of maltose	Percent of theoretical	*Mean*	Milligrams of maltose	Percent of theoretical	*Mean*	Milligrams of maltose	Percent of theoretical	*Mean*
140	130	92.9		127	90.7		127	90.7	
	131	93.6	93.1	127	90.7	90.5	129	92.1	90.9
	130	92.9		126	90.0		126	90.0	
224	207	92.4		204	91.1		193	86.2	
	205	91.5	91.5	204	91.1	91.1	196	87.5	87.2
	203	90.6		204	91.1		197	87.9	
280	255	91.1		261	93.2		245	87.5	
	253	90.4	90.4	261	93.2	93.2	251	89.6	88.2
	251	89.6		261	93.2		245	87.5	
336	298	88.7		316	94.0		292	86.9	
	296	88.1	88.3	316	94.0	94.0	293	87.2	87.4
	296	88.1		316	94.0		296	88.1	
Average		90.82			92.19			88.43	
Difference from mean of all methods		+.44			+1.81			−1.95	
Range of values		4.8			3.5			3.7	
Standard error, means of triplicate		1.92			1.53			1.76	
Standard error, within triplicate		.64			.20			.96	

tion reduction exactly 2 minutes. It was suggested by one collaborator that these two methods might be improved by adjusting the sugar tables to the use of a 30-minute digestion of 80° C. as specified in the Quisumbing-Thomas method.

The mean of the standard errors between collaborators for all methods was for samples 1, 2, 3, and 4; 5.49, 6.86, 9.79, and 10.93 respectively. This possibly would indicate that with a malt treated sample such as sample 4, results will not be as consistent between laboratories as with untreated samples or that greater variability between laboratories might be expected in samples of higher diastatic values.

Collaborator A made a detailed variance analysis of the grouped data for each method which is summarized in Table VI. The most satisfactory method for determining reducing sugars is the one which gives the smallest differences between collaborators, the greatest differentiation between flours, the most consistent results, *i.e.*, the lowest interaction for flours × collaborators, and the lowest differences between replicates, *i.e.*, the smallest experimental error.

Considering first the differences between collaborators, the variances given in Table VI show that the differences between the mean values for collaborators are greatest in the instance of the Blish-Sandstedt method and least for the Bertrand, and the relative differences, *i.e.*, the ratio of the variances for "between collaborators" to the corresponding differences "between replicates" are also in the same order. From Table II it will be seen that the means for collaborators (all flours) range from 234.2 to 252.2, or 18.0 units, for the Blish-Sandstedt method; from 239.0 to 257.4, or 18.4 units, for the Schoorl; and from 231.5 to 254.2, or 22.7 units, for the Bertrand. From these spreads it would seem that the Bertrand method gave the greatest differences between collaborators, but it must be remembered that the distribution of the samples along the ranges influences the magnitude of the variances. Thus, for the Blish-Sandstedt method, the collaborators tend to be grouped more definitely into those securing low and those securing high results, than in the instance of either the Schoorl or Bertrand methods.

Considering next the variance for interaction between collaborators and flour, it will be noticed that for all three methods, the collaborators secured different relative results with different flours, the interaction variance being highest with the Blish-Sandstedt method and lowest with the Schoorl. The F value for the interaction variance is, however, lower than for the Schoorl method, due to the higher experimental error. It is interesting to note that the pooled experimental error (differences between replicates) is lowest for the Blish-Sandstedt method and highest for the Bertrand. The error and interaction variances have been com-

TABLE VI

ANALYSIS VARIANCE OF METHODS

(Calculations made by Collaborator A)

Variance due to	DF	Variance	F	5% point	F_2	5% point
Blish-Sandstedt method						
Between collaborators	7	613.33	308.34	2.18		
Between flours	3	106,727.3	53,656.1	2.76	829.6	3.47
Interaction, flour × collaborators	21	128.65	64.68	1.70		
Between replicates	64	1.9891				
Total	95					
Standard error of single determination	1.31					
Schoorl method						
Between collaborators	7	581.68	179.0	2.18		
Between flours	3	128,445.70	39,521.8	2.76	1639.2	3.47
Interaction, flour × collaborators	21	78.36	24.1	1.70		
Between replicates	64	3.2500				
Total	95					
Standard error of single determination	1.803					
Bertrand method						
Between collaborators	7	551.94	101.9	2.18		
Between flours	3	117,042.6	21,605.7	2.76	1119.2	3.47
Interaction, flour × collaborators	21	104.58	19.3	1.70		
Between replicates	64	5.4172				
Total	95					
Standard error of single determination	2.327					

F = Variances compared with variance for between replicates as error

F_2 = Variances compared with variance for interaction as error

Comparison of corresponding variances

F values

Variances compared	Blish-Sandstedt vs. Schoorl	Schoorl vs. Bertrand	Blish-Sandstedt vs. Bertrand	5% (approx.)
Interaction	1.64	1.33	1.23	2.1
Replicates	1.63	1.67	2.72	1.5

pared directly, and the results recorded at the foot of Table VI. It will be noted that the experimental error for the Blish-Sandstedt method is significantly lower than for both the Schoorl and Bertrand procedures, and the Schoorl significantly lower than the Bertrand.

The Blish-Sandstedt method, while giving the lowest actual variance for "between flours," gives greatest differentiation in comparison with standard error. The narrower spread between flours by this method has already been referred to, the range for flours being from 167.9 to 308.9, or 141.0 units, as compared with 165.4 to 318.2, or 152.8 units, for the

Schoorl method, and 165.7 to 311.8, or 146.1 units, for the Bertrand method.

In relation to experimental error, however, the Blish-Sandstedt method gives the highest F value and therefore the best differentiation. Taking the figures at their face value, the data indicate that the Blish-Sandstedt method will give the best differentiation between flours for any one laboratory. However, the experimental error does not seem to be the proper one to use for comparing the differentiation between flours when considering the combined data for all collaborators. The differentiation between flours, in this instance, depends upon not only the experimental error, but also upon the uniformity in the results obtained in different laboratories, *i.e.*, upon the interaction of collaborators × flours. The interaction variance also contains the replicate variance and is the only logical variance to use in comparing the relative value of the three methods for general use. The method which gives the most consistent values in different laboratories on different flours is obviously superior for general use to one which gives less uniform results in different laboratories even though the laboratory error is somewhat higher. The F_2 value for "differences between flours" is very much higher for the Schoorl than for the Blish-Sandstedt method and must therefore be regarded as superior for general use.

The results on maltose (sample No. 5, Table IV) lead to about the same conclusions as those reached from a study of the data obtained on the flour samples. The Schoorl method gives the highest, and the Blish-Sandstedt the lowest values; while the experimental error is lowest for the Blish-Sandstedt and highest for the Schoorl method. The standard error for between laboratories places the methods in the same relative order as the experimental error or standard error within triplicates.

Indications are that the small variations in pH of buffer solutions used by these collaborators had no marked significance on the results. For example, chemist B with the highest pH value, reported the lowest mean value for all methods and this collaborator also reported the lowest corresponding mean value on sample No. 5 in which the buffer solution had no effect on the results. Chemist D used a buffer solution of the lowest pH value and the mean value for all flours tested by all methods was close to the average for all collaborators.

Summary

The Blish-Sandstedt, Schoorl, and Bertrand methods for measuring the reducing sugars after diastasis in the diastatic activity test of flour, are about equally satisfactory. A slight adjustment in the tables of these three methods in the higher sugar levels would make the values

more comparative. The merits of the three methods as indicated by the analysis of the data from this collaborative study would rate them for general use as follows: first, Schoorl; second, Blish-Sandstedt; and third, Bertrand. Because of the greater uniformity between laboratories and a greater differentiation between flours, the Schoorl method is preferable even though it has a higher experimental error than the Blish-Sandstedt method. The Bertrand method was rated last, primarily because of its higher experimental error. Most collaborators were unfamiliar with the titration in this method and did not like the end-point or stated that more practice would possibly have made the results more comparative. One collaborator regarded the Schoorl and Bertrand methods as preferable, because other sugars could be measured with the same procedure. Most collaborators preferred the Blish-Sandstedt method because the ferricyanide reduction in a boiling bath was relatively simple and the experimental error was low.

REFRACTOMETRIC METHODS OF DETERMINING DIASTATIC ACTIVITY OF FLOUR [1]

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(Received for publication August 24, 1936)

Molin (1934) proposed a rapid refractometric method for estimating the degree of hydrolysis of a flour-starch-water preparation at an elevated temperature. Flour was mixed with 2.4 times its weight of water and the mixture incubated in a thermostat for exactly 10 minutes at 62° ± 0.1° C. The preparation was quickly cooled, sufficient water added to bring the flour : water ratio to 1 : 4, allowed to settle, filtered and the refractive index determined at 17.5° C. by means of an immersion refractometer having a prism of $n_D = 1.325 - 1.366$. A blank determination was made with the same flour without incubation to provide the necessary correction in order to estimate the change in n_D in consequence of incubation at the elevated temperature.

Doubtless a variety of solutes contribute to the n_D of such preparations, although it appears reasonable to believe that soluble carbohydrates are most prominent in this connection. Moreover, the changes which occur during such treatments as are thus accorded flour may be due to enzymic hydrolyses, and also to the hydration effect of the water at the relatively high temperature employed. In

[1] Paper No. 1412, Journal Series, Minnesota Agricultural Experiment Station. Condensed from a thesis presented by Emil Munz to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1935.

an attempt to demonstrate the effect of temperature alone, we treated Merck soluble starch, Merck dextrin, and a gelatinized starch preparation with water at 60° C. for 15 minutes, with the results recorded in Table I. The refractive index was measured with a Zeiss immersion

TABLE I

EFFECTS OF TREATING SOLUBLE STARCH, DEXTRIN AND GELATINIZED STARCH IN THE PRESENCE OF WATER AT 60° C. FOR 15 MINUTES

Material used		Refractive index in degrees as registered on the immersion refractometer	Calculated sugar if n_D was due to sugar alone	Reducing sugars by Fehling's reduction		Sugars by Blish gassing method
				As glucose	As maltose	
		n_D	%	%	%	%
Soluble starch	Before heating	15.35	1.7	0.35	0.55	1.2
	After heating	22.30	21.0	1.50	2.23	2.2
Dextrin	Before heating	36.25	57.5	5.17	8.73	7.4
	After heating	45.05	78.5	5.94	10.04	7.4
Gelatinized starch	Before heating	18.00	—	—	—	—
	After heating	20.30	—	—	—	—

refractometer both before and after the heat treatment. In addition, the sugars present were estimated by Fehling's reduction, and by the Blish gassing method in the instance of the soluble starch and dextrin. We also computed the sugar that would be present in the event that the refractive index was due to it alone. These estimates, when contrasted with the reducing sugar determinations, indicate that substances other than sugars produced by hydrolysis are responsible for much of the change in n_D that results from this heat treatment.

Effect of Flour : Water Ratios

It was not fully apparent from Molin's published work as to exactly why he adopted the particular conditions involved in his technique. Since we proposed to employ a refractometric method extensively in the course of certain researches it appeared desirable to determine the effect of several variables before standardizing our procedure. The flour-water ratio was first studied, and ratios ranging from 100 : 80 to 100 : 1000 were included. A digestion period of 15 minutes at 65° C. was chosen. To insure a variable rate of hydrolysis of flour constituents in each instance, wheat malt was added to a normal bread flour in proportions ranging from 0.125% to 2% by weight of the flour. No blank determinations were made and the observations are recorded in Figure 1 as they were read directly on the immersion refractometer scale after correcting to the same dilution. The least dilution used was

closest to the consistency of an ordinary bread dough in which 60 to 65% of water is usually employed. The highest dilution of 100 : 1000 is the same as is involved in the Rumsey diastatic activity method.

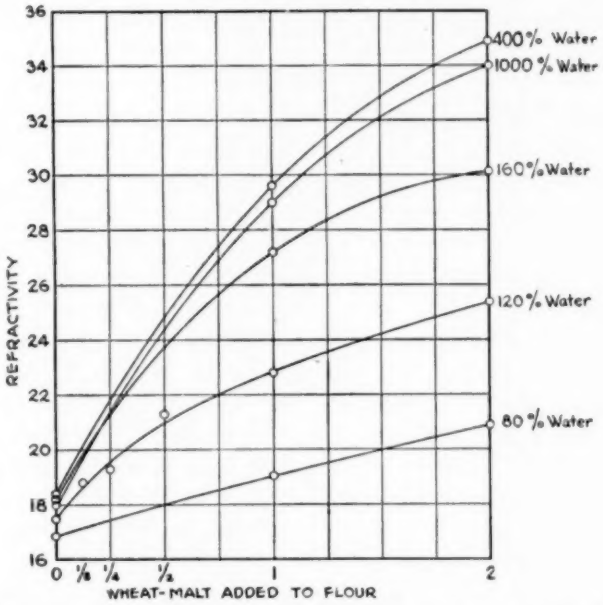

Figure 1. Influence of water concentration (in terms of flour) on refractive index of digestates held at 65° C. for 15 minutes, and with all refractive indices finally measured at the same dilution.

From the graphs it is apparent that dilutions progressing from 100 : 80 up to 100 : 400 and digested at 60° C. result in a gradual increase in the refractive index when wheat malt is present in the mixture. In another series not recorded here, the digestion was conducted at 50° C., and there was little difference in n_D when the flour-water ratio was stepped up from 100 : 120 to 100 : 400. Accordingly, it appears that at the more elevated temperature additional water within certain ranges either directly factilitates diastase action or more probably aids in hydrating the amyloses so that they constitute a more labile substrate. When the ratio of flour : water was further increased to 100 : 1000 there was a tendency toward a somewhat diminished production of refracting solutes in these preparations.

The effect of dilution was further studied using both patent and clear flours reinforced with wheat malt. In both instances the refractive index increased with dilution up to a flour : water ratio of 100 : 600. Moreover the increase in n_D progressed regularly with the increase in maltose content when the latter was determined by the Schoorl method. Greater dilution up to 100 : 1000 and 100 : 2000

resulted in no substantial change in n_D but did result in a maltose : n_D ratio that was narrower than resulted with the lower dilutions. Accordingly it was concluded that flour : water ratios in the range of 100 : 400 to 100 : 500 were probably optimum for such refractometric estimates of hydrolysis in flour-water suspensions.

Effect of Buffer Salts

In many of the earlier studies of diastatic activity an effort has been made to bring the H-ion concentration to a definite and known level, and maintain it there by means of buffer solutions. Sorenson's citrate buffer has been employed in this manner in certain flour diastase determinations. To ascertain what effect such a buffer would have on the changes in refractive index of autolysed flour-water mixtures, a series of preparations was made with varying amounts of wheat malt up to 2%, and adjusted with citrate buffer to pH 5.1, and pH 6.2. The unbuffered mixture was also included. With a flour-water ratio of 100 : 400 and 2% wheat malt, the refractive index after autolytic digestion was n_D = 22.3° at pH 5.1; n_D = 25.1° at pH 6.2, and 34.2° in the unbuffered mixture which had a pH somewhere between these extremes. In other experiments in which the pH was adjusted through the use of a higher dilution of the buffering salts, the value of n_D rose to somewhat higher levels.

Evidently the salt ions of these buffering solutions depressed in some manner the rate of hydrolysis. It is known that the swelling of starch granules is an inverse function of the osmotic pressure of aqueous solutions in which they are suspended. This may result in a diminished diastatic lability of such granules when buffering salts are used at the elevated temperature employed in this refractometric method.

Electrolytes of the buffers may also exert an influence upon the physicochemical properties of the colloid complex represented by amylase-starch. There may be a more direct depressing effect of the salt ions upon the enzyme itself, although this has not been so apparent in previous work, and particularly when the buffer salts tended to increase the H-ion concentration in the direction of pH = 4.7.

Effect of pH at Elevated Temperatures

A highly diastatic bread flour was employed in this phase of the work, and accordingly it did not require any supplementing with wheat malt. To avoid the effect of buffer salts, the adjustments of pH were made by the direct addition of 0.01 N lactic acid. The range represented was from pH 5.85 to pH 4.42.

Autolysis was measured in terms of maltose production as determined by the Schoorl reducing sugar method, and by the refracto-

metric method. Temperature as a variable was also introduced, the range employed being from 30° C. to 65° C. Two ratios of flour to water were included, 100 : 400 and 100 : 900, but the results were so similar that only the narrower ratio is reported here. In Figures 2 and 3 appear graphic records of these studies and it is apparent that the

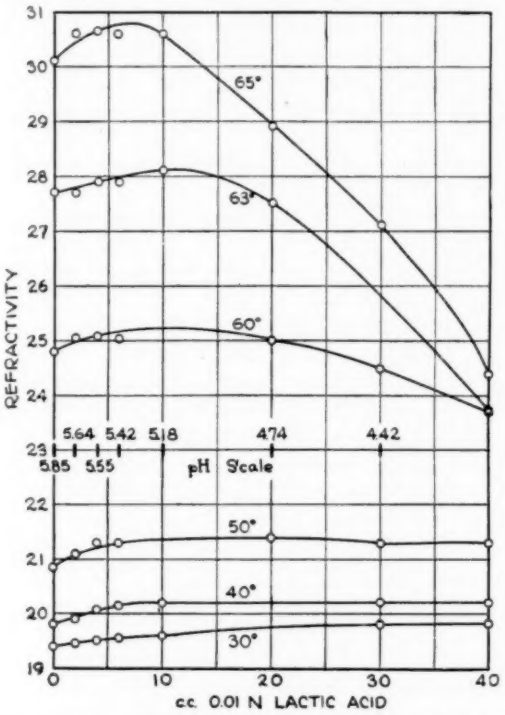

Figure 2. Diastatic activity as measured in terms of refractive index, at different levels of temperature, and acidity. Digestate consisted of 100 parts of flour to 400 parts of water, and held at the respective temperatures for 15 minutes.

region of maximum hydrolysis as a function of pH tends to shift in the direction of a lower H-ion concentration as the temperature is elevated from 30° C. to 65° C. In general, this is in harmony with the earlier observations of Olsen and Fine (1924) rather than the opposing views of Lüers and Nishimura (1926).

Influence of Flour Grade and Ash Content

Since electrolytes contributed by buffer salts depressed the rate of increase in n_D of autolysed flour suspensions, it appeared possible that variations in the content of electrolytes of natural flours might become a variable likewise. To test this possibility, aliquots of a patent flour were mixed with 10%, 20%, and 30%, respectively, of a clear flour and

the refractive index was measured after autolytic digestion with 400% of water for 15 minutes at 63° C. and 65° C. Since the clear flour was probably higher in diastatic activity than the patent, 0.5% of wheat

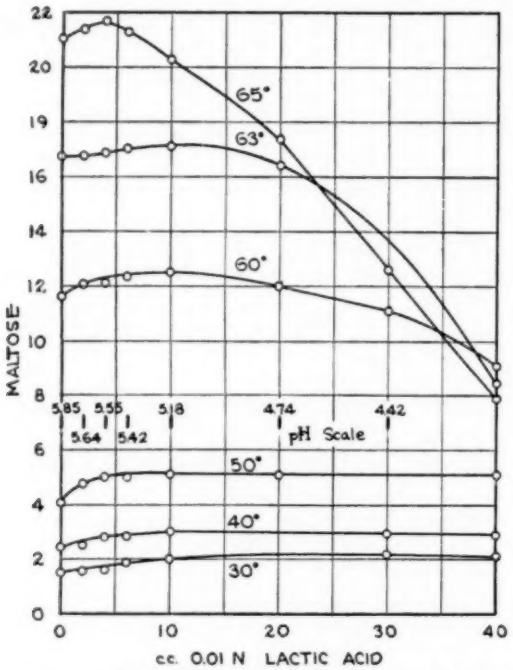

Figure 3. Conditions identical with Figure 2 except that the diastatic activity was measured in terms of maltose produced.

malt flour was added to minimize this difference. The refractive indices of these digestates were practically identical with that of the pure patent flour, although the ash content had been increased from 0.40% in the patent to .58% in the mixture with 30% of clear flour. Thus, it appeared that the increase in electrolyte content that must have been effected by the addition of substantial proportions of clear flour had no effect upon the n_D of such digestates.

Influence of Temperature

Molin digested his flour suspensions at 62° C., which approaches the temperature at which Ohlsson effected the inactivation of β-amylase or saccharogenamylase in 10 minutes, namely 70° C. The α-amylase or dextrinogenamylase is reasonably stable under such treatment. It appeared desirable to explore this thermal effect somewhat more fully and a series of experiments was accordingly conducted in which the flour was autolysed for 15 minutes at 30° C. and at temperatures vary-

ing by 5° C. intervals from 55° C. to 65° C. As in other studies mentioned above, 0%, .125%, .25%, .5% and 1% of wheat malt flour were used in the mixtures. Not only did the refractive index increase progressively as the temperature was elevated through this range, but the effect of the added malt was also emphasized as is evident from the slope of the graphs in Figure 4.

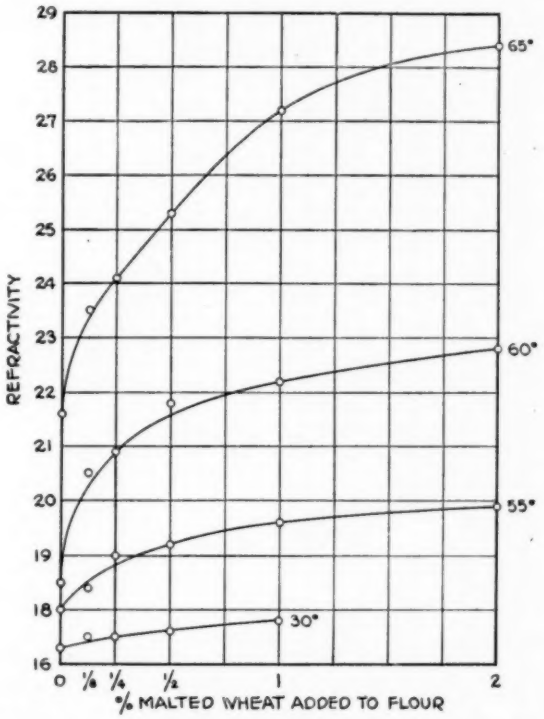

Figure 4. Diastatic activity as measured in terms of refractive index, and varied by the percentage of added malted wheat flour, and temperature of digestion. All preparations digested 15 minutes.

This initial temperature study was hardly adequate to a determination of the most suitable temperature, however, and accordingly a more searching study was instituted with ten temperature levels ranging from 56° C. to 70° C. Only one flour was used, a bread flour having normal diastatic activity, and the flour-water ratio was 100 : 500. The time of digestion was introduced as an additional variable to disclose whether or not the rate of autolytic hydrolysis was relatively constant as a function of time. Both refractive index and maltose production were determined in the instance of these preparations. The results are so significant as to justify several approaches to the analysis of the extensive data that were accumulated.

In Figure 5 the refractive index is recorded on the vertical axis, time in minutes on the horizontal axis, and graphs were drawn for the data accumulated at each level of temperature. The graph for the 70° data started somewhat above that of the 68° series but the two merged after 25 minutes. Apparently those materials which contribute to n_D of such preparations appear at a greater rate as the

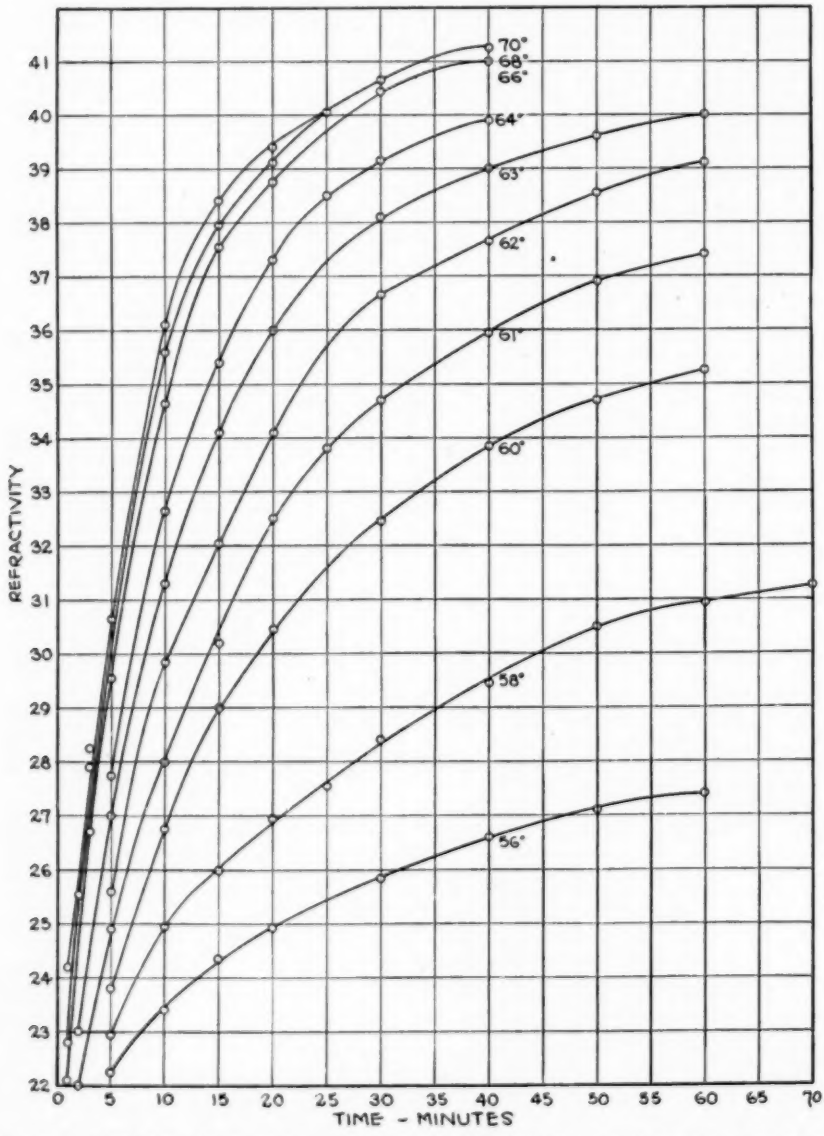

Figure 5. Diastatic activity as measured in terms of refractive index, and determined over various time intervals at temperatures ranging from 56° C. to 70° C.

temperature is elevated to 68° C. or 70° C. That all of these substances are not sugars is suggested by the maltose content of the same preparations, which is recorded graphically in Figure 6. It is evident that when the temperature of digestion exceeded 64° C. the relative rate of increase in reducing sugar content was not as great as the rate of increase in n_D. In other words, refractive materials other than reducing sugars appear progressively on protracted digestion at 66° C. to 70° C.

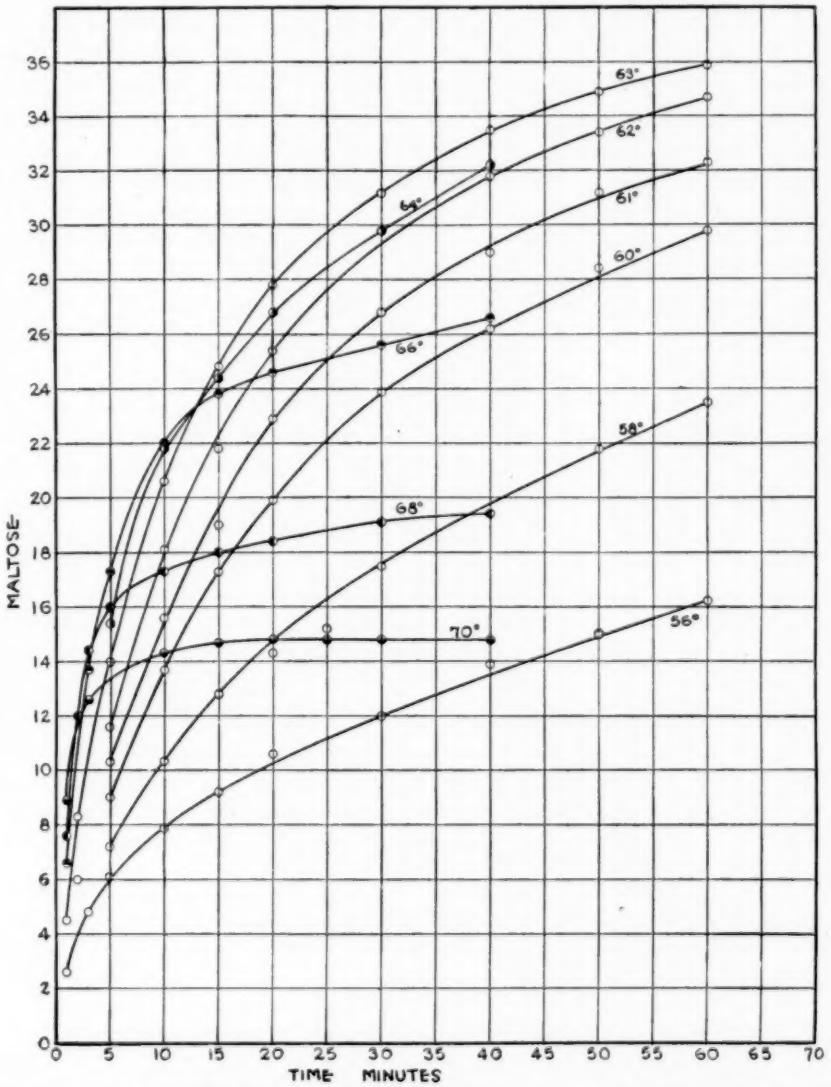

Figure 6. Diastatic activity as measured in terms of maltose production and determined over various time intervals at temperatures ranging from 56° C. to 70° C.

This relation between the rate of change in n_D and maltose content as a function of temperature is better demonstrated by the graphical record of the same data in Figure 7. An approach to a linear relation between n_D and percentage of maltose appears in the instance of the preparations digested at 62° C. or 63° C. Above 63° C. the refractive

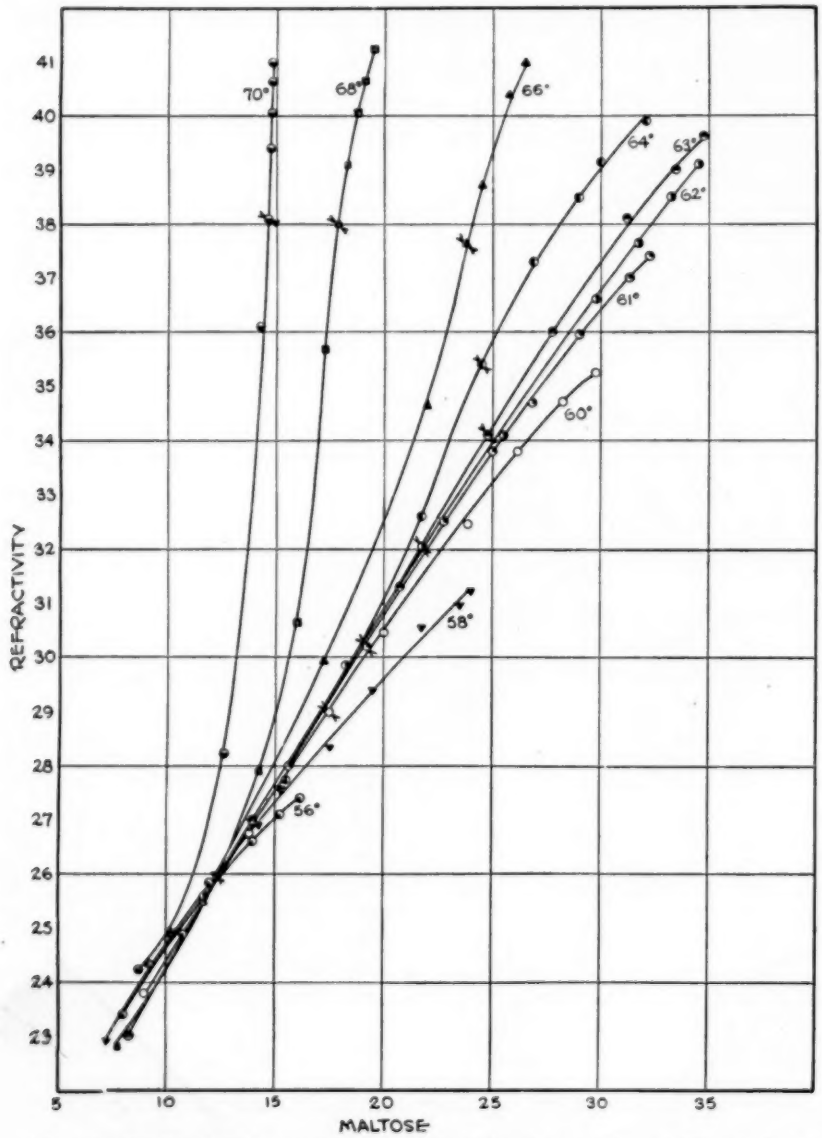

Figure 7. Relation of maltose content to refractive index of preparations of 100 parts flour to 500 parts water digested for varying time intervals (with the 15-minute observation indicated by arrows in each case) at temperatures from 56° C. to 70° C.

index is greater than would be anticipated from the reducing sugar content after the first few minutes of autolysis. Accordingly, the digestion temperature employed by Molin, 62° C., is apparently optimum for this type of measurement. Less error results from digestion at lower temperatures than from temperatures above 64° C.

Variability of Replicated Measurements

To begin with, the range in n_D of natural flour digestates held at 62° C. for 15 minutes is relatively small, being only about 6° on the immersion refractometer scale, between ordinary low diastase flours and the general run of natural flours of normal diastatic activity. Moreover, the variability of the initial experiments was so great as to necessitate 3 to 5 replicate determinations at all temperatures over 55° C. It accordingly appeared advisable to attempt a refinement of the technique to reduce the variability as much as possible and thus minimize the extent of replication that would be necessary.

A study of the effect of flour temperature upon the final n_D indicated the desirability of standardizing the preheating of the flour, and accordingly the latter was made uniform by incubating the flour in the dry state at 30° for a sufficient time so that it attained that temperature.

Other variables were progressively introduced in an orderly fashion, and the standard deviation (σ) of a series of 10± replicated determinations was computed in each instance. The least variability ($\sigma = 0.114$) was encountered in 10 replicated determinations conducted as follows: 25 cc. of water was brought to 62° C. in a test tube 2.1 cm. inside diameter by 20 cm. long; 10 g. flour was introduced through a funnel and suspended by vigorous agitation for 10 seconds. The tube containing the suspension, tightly closed with a rubber stopper was then submerged in a 62° C. water thermostat and kept for exactly 15 minutes. Then 22.5 cc. of a solution containing 0.3 g. of sodium tungstate followed by 2.5 cc. of 10% H_2SO_4 were added successively with shaking. It was then cooled rapidly by submerging in a thermostat at 18° C. which materially slowed down the amylolysis. When cooled it was filtered rapidly through an S. and S. No. 588 folded filter and the n_D promptly observed.

This method was then applied to duplicate determinations with 10 different flours. The maximum difference between duplicates was 0.24° (immersion refractometric scale) and the average difference was only 0.12°.

Summary

Flour : water ratios in the range of 1 : 4 to 1 : 6 were best adapted to the refractometric method for the measurement of the autolytic hydrolysis of wheat flour. The ratio of maltose content : n_D of the

digestates became narrower when the flour : water ratio was greater than 1 : 6.

Salts contributed by citrate buffer solutions apparently reduced the rate of increasing n_D of digestates below the rate at equivalent pH levels when the latter was adjusted by adding lactic acid solutions. Buffering materials contributed by low grade flours do not affect the n_D values appreciably, however.

Temperature of digestion of flour suspensions in water influences the maltose : n_D ratio. A linear ratio is approached when the flour suspensions are incubated at 62° C. to 63° C. At higher temperatures there is a substantial departure from linearity, the n_D increasing faster than the maltose content.

Variability of replicated measurements of n_D was computed in the instance of several series in which variables in the techniques were progressively modified. This afforded a basis for selection of one technique, which provided adequate and convenient chemical and physical conditions and at the same time was least variable. This technique, when applied to duplicated measurements of autolytic hydrolysis in the instance of 10 different flours, resulted in good agreement between the duplicates.

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WHEAT QUALITY IN THE SOUTHWEST AS VIEWED BY THE AGRONOMIST

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(Read at the Annual Meeting, June 1936)

Quality is an elusive thing. It is almost as difficult to define as it is to attain. It varies with the viewpoint.

One of the large flour mills in Kansas includes in all its advertisements the statement that "Kansas grows the best wheat in the world." Without casting any aspersions on Kansas wheat, and admitting freely that like the wheat of Oklahoma, Nebraska, and Texas, it is unexcelled for certain purposes, I still wonder whether a pretzel baker would subscribe to this glowing statement, or what throes of agony a cake manufacturer would undergo in attempting to make a choice angel-food cake from the hard red winter wheat flour of western Kansas.

The very fact that the A. A. C. C. has various committees on cake, biscuit, cracker and pie flours, is evidence enough for anyone that different kinds of wheat are required for different purposes, and that the factors which constitute quality under one set of conditions may be the factors which indicate a lack of quality under a different set of conditions.

Of course, this is common knowledge to all of you, and I emphasize it only to bring out this one point: You, as cereal chemists, have widely different conceptions of quality in wheat depending upon the nature of the product with which you are chiefly concerned. But the differences in your viewpoints as to what constitutes quality are not nearly so great as the divergence between the viewpoints of the man who grows the wheat and the man who processes it.

The agronomist lies somewhere between the grower and the processor, and if he is a good agronomist he will know something about the problems of both. It is true that agronomists in the past have sometimes ignored the problems of the miller and baker, and have distributed to farmers new varieties of wheat which had certain objectionable features, so far as milling and baking properties are concerned. On the other hand, the milling industry has sometimes objected strenuously to new varieties, apparently simply because they were new and different. Fortunately this situation is changing.

What is quality in wheat from the growers viewpoint? I should

say that the farmer would consider a wheat as having quality if it has proven its ability to produce a crop in spite of the hazards of insects, weather, and diseases. Let us consider some of these hazards and discuss some of the efforts of agronomists and others to surmount them.

Drought

Much of the wheat in the southwestern states is grown in areas of low rainfall. It is, indeed, this very fact which gives the hard red winter wheat of the Great Plains region the characteristics which have made it among the wheats *par excellence* for modern commercial bread-making. Our southwestern soils are high in nitrates and other plant nutrients because there has never been sufficient rainfall to leach these elements from the soil. Protein in wheat is nothing more than nitrogen from the soil converted into nitrogen in the grain and we cannot produce a high protein wheat unless we have an abundance of available nitrates in the soil. But we cannot produce any wheat at all unless we have sufficient water in the soil and water is usually the limiting factor in the southern part of Great Plains region. The average annual rainfall, usually around 20 to 25 inches, is ample for crop production if it were all available, or available every year. Unfortunately, as the rainfall decreases in amount, it increases in variability so that we may have seasons with far more rain than is needed and others with far less. Then, too, there seems to be a tendency for the weather in the Great Plains to follow cycles. These cycles are not well defined and our weather records are not long enough to permit us to predict when they will occur, but few people familiar with conditions in the Great Plains region have any doubt that they do occur. Like Joseph, they know that the fat years will be followed by lean years. Our famous "dust bowl" has been going through the "lean year" period but, make no mistake, the "lean" years will come to an end and will be followed by "fat" years.

It may be that the cycle of good years is just beginning. The Texas Panhandle has reported rains of almost record proportions during the past few weeks. Whether this means the end of the "dust bowl" remains to be seen, but sooner or later normal rainfall will occur and the "dust bowl" will be forgotten until another cycle of dry years strikes the sub-humid Great Plains region.

In the meantime, is there anything that can be done to reduce the ravages of drouth? R. E. Dickson, Superintendent of our Substation at Spur, has found that of the 22 inches of rainfall which occur in that region, approximately one-fourth is in the form of small showers which are lost through evaporation without being effective in crop production. Another 25 per cent is lost through run-off. In other

words, we have been attempting, with a rather surprising degree of success, to produce crops with only about 11 inches of effective rainfall. If the 5½ inches lost through run-off could be conserved, and we know that it can, through terracing and other methods of management, wheat growing would be confronted with fewer hazardous years on account of lack of moisture. Millions of acres of land in West Texas and other parts of the Great Plains region have now been terraced or listed on the contour and at the present rate the entire Great Plains region will be terraced before many more years have elapsed. When this is accomplished wheat production will be a far more stable enterprise in this region than it has ever been before.

Another practice that is being recommended, but one which so far has but a small following, is that of "opportunity farming." Extensive records in Kansas, Oklahoma, and other states have shown that when the moisture content of the soil in the fall at seeding time drops below a certain minimum, the odds against producing a profitable crop the following spring are overwhelmingly large. Under these conditions it should pay the farmer to forego planting wheat, knowing that his chances of making a crop are ridiculously small. But all farmers are gamblers; the whole business of farming is a continual gamble with weather, insects, and diseases, and most southwestern farmers would prefer to take a chance against large odds on making a crop, rather than let their land lie fallow only to be certain of making no wheat at all.

Finally, the plant breeder may be of some help in overcoming drouth by developing new varieties capable of withstanding droughty conditions. Varieties of corn grown in the southwest are far more resistant to drought than those grown in the corn belt, and there is no doubt that the drought resistance of our southwestern wheat varieties could be improved by hybridization and selection. We have been handicapped in undertaking work of this nature because drought resistance is an intangible thing and very difficult to measure under field conditions. The work of Canadian agronomists in developing artificial drouths in the laboratory, and the recent discovery by Dr. Bayles of the United States Department of Agriculture, that drouth resistance is apparently associated with the rate of water loss from leaves removed from the plant, both furnish guides for future research and I hope that this problem will receive considerable attention in the near future.

Winterkilling

Practically all the wheat in the southwest is winter wheat and must, in fact, go through a winter before it is capable of heading. During the process it is sometimes killed by extremes of cold weather.

The most promising solution of this problem is one of breeding winter hardy wheats. In some seasons there is no winterkilling whatever, while in others all the wheat may be killed. In the past it was often difficult for a plant breeder to obtain information on the winter-hardiness of his selections. Today, however, he no longer depends on nature's vagaries to tell him how cold resistant his different strains are. He puts them in a specially designed refrigerator and freezes them to determine how much hardiness they have. Excellent work along these lines is in progress at the Kansas, Nebraska and Minnesota Experiment Stations. In Texas we are less concerned with cold resistance in wheat as most of the varieties now grown survive our milder winters with but little loss.

The Russians have recently discovered or re-discovered a method of treating winter wheat so that it may be planted in the spring. This treatment known as "yarovisation" or "vernalization" is used when the fall planting has been killed during the winter. The Russians are claiming great success for this method but experiments in this country indicate that spring wheat varieties planted in the spring will make more wheat than the yarovised winter wheat varieties planted in the spring. This being true, the wheat breeder will continue to consider winter hardiness as a factor in wheat quality and will distribute only those varieties which are able to survive the winter freezes.

Diseases

"Wheat may survive the drought and freezes only to succumb to a host of diseases." The rhyming is not intentional and in spite of the rhyme there is much truth in the statement. Last year at our Sub-station at Denton, wheat varieties almost ready to cut, that looked good for a yield of thirty bushels per acre, actually made a yield of about three bushels when finally harvested and threshed. This loss of nine-tenths of the crop was brought about by a sudden and devastating epidemic of stem rust which spread like wild fire, and, like wild fire, left destruction in its wake. Starting in North Texas the rust epidemic spread northward into Oklahoma, Kansas, and Nebraska, literally wiping out millions of dollars worth of wheat, leaving the wheat farmer with a miserable crop of shriveled wheat with a test weight of 45 to 50 pounds per bushel. Here is one place where the farmer and the miller see eye to eye when it comes to the question of quality. Neither one is interested in wheat only for the bran that it produces. What can, and what are, the agronomists doing about rust? There is only one thing to be done; develop rust resistant varieties. There is no seed treatment which will prevent rust in wheat because the organism which causes the disease is not borne in, or on, the seed. There is no very

satisfactory method of controlling rust after it has made a start, though the Canadians are doing some experimental work on controlling rust by air plane dusting with sulphur. This leaves plant breeding as the only promising recourse. Fortunately, several rust-resistant varieties are now available as parental material and plant breeders in all the southwestern states are using these in their breeding program. Fortunately for the farmer, but unfortunately for the plant breeder, stem rust does not occur every year so that it is not always possible to identify and isolate the rust resistant segregates.

It so happens that we have at College Station, the headquarters of the Texas Experiment Station, a combination of natural conditions which produces a severe rust epidemic every year. Our winters are mild so that rust often over-winters and infection starts early in the spring. Spring rainfall is abundant and the proximity to the Gulf results in frequent mists and heavy dews. All of these factors contribute to such an extensive development of rust that ordinary varieties of wheat cannot be grown at all, and there has never been any wheat production on farms in this locality. But the very same hazards which have made commercial wheat production impossible in this locality, make it ideal as a place to breed for rust resistance. Any wheat which will survive the severe rust epidemics which occur regularly in South Texas, is almost certain to withstand ordinary epidemics in the wheat belt proper. The United States Department of Agriculture and the Texas Experiment Station are cooperating in using this locality as a testing ground for wheats from all the southwestern states, and also in developing new rust resistant varieties which will serve as a barrier against the spread of rust to the northern states. Encouraging progress has already been made. Last year a number of the new wheats which showed resistance under South Texas conditions went through the severe epidemic in Kansas and Nebraska without a trace of rust. It appears now that the rust problem can be solved.

Another disease that is coming under control is stinking smut, or bunt. Conditions in the Southwest are very conducive to the development of this disease and were it not kept under control through seed treatment with copper carbonate, the losses from stinking smut would be truly enormous. Considerable progress is now being made in developing varieties which are immune to this disease. Dr. Quisenberry, who follows me on this program, has been very active in the development of smut-resistant varieties and will probably tell you in detail the progress that has been made. Suffice for me to say that smut resistance is inherited as a Mendelian character and it is possible, by a system of breeding known as back-crossing, to incorporate smut resistance into our present varieties without changing them appreciably

in any other way. In other words we can add some factors for quality from the grower's standpoint without changing or losing the factors for quality from the miller's standpoint.

There are many other diseases to which wheat is heir but enough has been said to show their importance as factors in quality.

Earliness

Earliness is important from many standpoints. Several days in earliness may mean the escape of rust, drought, or hail. With the widespread use of the combine harvester throughout the Southwest, earlier varieties also need to be grown along with main season varieties to spread out the harvest period. Here again there is little in the way of cultural methods that will increase earliness and plant breeding is the most promising method of attack. Dr. Parker, of the Kansas Station, who has probably done more work on this problem than any other agronomist, has succeeded, by using early varieties from Australia as parental material, in developing new hard red winter wheats which are considerably earlier than the varieties grown at present. If Dr. Parker were an agronomist familiar only with the problems of the grower, these new wheats would undoubtedly be in the hands of farmers today. But he, probably more than any other wheat breeder in the country, has kept in close touch with the cereal chemists and and with the problems of the milling industry, and no new wheat receives his blessing until it has run the gauntlet of repeated tests by cereal chemists throughout the country. I sometimes wonder if he is not too critical of the progeny of his plant breeding art, especially since cereal chemists have been known to disagree regarding the merits of new varieties, but in any case you may rest assured that when his new early wheats are distributed to the farmer, they will have, not only those qualities which the grower so earnestly desires, but also the qualities which the miller requires.

One might go on almost indefinitely in this same vein, speaking of the work that is being done on other factors which make for quality from the grower's standpoint; resistance to shattering, resistance to lodging, resistance to injury from Hessian fly, and other characteristics too numerous to mention, but it would make a long story. Let me complete the story in a nut shell by stating that wheat quality, as viewed by the southwestern agronomist, includes those characteristics which make a wheat variety capable of surviving the hazards of weather, insects, and diseases, and at the same time capable of standing up under the processes of modern milling and baking. To attain this quality the agronomist must work with many wheats; he may get earliness from Australia, rust resistance from Russia, stiffness of straw

from Italy, but always he must strive to maintain those milling and baking characteristics which have made the hard red winter wheats of the Southwest famous as bread wheats throughout the world.

ON THE CHANGES OF LEUCOSIN, GLIADIN, AND GLUTENIN UNDER THE ACTION OF FLOUR AND YEAST PROTEINASES

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(Received for publication April 23, 1936)

In our previous paper (Blagoveschenski and Yurgenson, 1935) we described our investigations of the action of enzyme mixtures from wheat flour and yeast on the protein mixture contained in gluten. It is of interest to investigate the breakdown of single gluten proteins under the action of enzyme extracts from flour and yeast, and under the action of pure proteinases free from other proteolytic enzymes, such as polypeptidases and dipeptidases.

For our experiments we prepared three protein preparations—leucosin, gliadin, and glutenin—by the successive extraction of wheat meal with water, 70% alcohol, and 0.2% sodium hydroxide solution according to Osborne's classical methods (Osborne and Voorhees, 1893; Osborne and Campbell, 1900).

The flour enzyme preparations were made by extracting 10 g. of flour with 100 mm. of M/6 acetate buffer at varying pH at 37° C. for 24 hours in the presence of toluene. For the experiments, 0.1 g. of the protein was mixed with 20 mm. of acetate buffer solution, 5 mm. of enzyme extract, and 1 mm. of toluene. For the control experiments the protein solution was mixed with 5 mm. of buffer solution instead of enzyme extract.

We determined the amino-nitrogen by the Van Slyke method and the nitrogen of the substances not precipitated by trichloroacetic acid (Rest-N) by the micro-Kjeldahl method. The duration of proteolysis in all our experiments was 24 hours, the temperature was 37° C.

The results of experiments with individual proteins and mixtures of the proteolytic enzymes of flour are given in Table I.

The maximum increase of amino- and rest-nitrogen for leucosin splitting by wheat flour enzyme mixture falls within the pH interval of 3.7 to 4.7, for gliadin splitting at a pH 3.7, and for glutenin splitting at a pH of 4.9 to 5.3. Glutenin is more resistant to the action of

proteolytic ferments than leucosin or gliadin. It is clear from these experiments that the maximum increase of free amino-groups was found with leucosine, the minimum with glutenin, both absolutely and also relatively to the amounts of rest-nitrogen. We found the relation of amino-nitrogen to rest-nitrogen, in % of the latter, for leucosin 72.9, for gliadin 53.3, and for glutenin 23.1 (mean figures for all pH values).

TABLE I

THE ACTION OF FLOUR PROTEOLYTIC ENZYMES ON THE INDIVIDUAL PROTEINS
(*in milligrams of nitrogen*)

	Amino-N			Rest-N		
pH	Beginning	End	Increase	Beginning	End	Increase
			Leucosin			
3.2	2.22	6.40	4.18	9.00	15.40	6.40
3.7	2.62	7.67	5.05	9.05	15.90	6.85
4.7	2.20	7.20	5.00	8.92	15.95	7.03
4.9	2.10	7.00	4.90	9.06	15.70	6.64
5.3	2.01	6.80	4.79	9.10	15.70	6.60
5.6	1.98	6.47	4.49	9.00	15.10	6.10
6.2	2.00	5.00	3.00	8.90	12.40	3.50
			Gliadin			
3.2	1.25	3.94	2.69	7.25	10.50	3.25
3.7	1.42	5.48	4.06	9.42	15.73	6.31
4.7	1.28	4.71	3.43	8.07	12.42	4.35
4.9	1.20	3.98	2.78	7.01	11.20	4.19
5.3	1.12	2.78	1.66	5.81	10.60	4.79
5.6	1.03	2.31	1.28	5.41	10.30	4.89
6.2	0.80	1.28	0.48	4.60	7.50	2.90
			Glutenin			
3.2	0.83	1.00	0.17	4.44	5.30	0.86
3.7	1.01	1.35	0.34	4.98	5.90	0.92
4.7	0.96	1.42	0.46	5.36	7.20	1.84
4.9	1.07	1.69	0.62	5.36	8.10	2.74
5.3	1.00	1.60	0.60	5.25	8.04	2.79
5.6	0.95	1.53	0.58	5.25	7.70	2.45
6.2	0.78	0.90	0.12	4.35	5.20	0.85

The second series of experiments was made with the enzyme extracts from yeast (10 g. of fresh pressed yeast, 100 mm. of acetate buffer in the presence of toluene at 37° C., autolysis during 24 hours).

These results and those obtained with wheat flour enzymes were very similar. The maximum increase of amino- and rest-nitrogen was found for leucosin to be at a pH of 3.7 to 4.7, for gliadin at a pH of 3.7, and for glutenin at a pH of 4.9 to 5.3. The glutenin was also more resistant than gliadin and leucosin to the action of yeast proteolytic enzymes, and the splitting of leucosin is the greatest both absolutely and relatively. The relation of amino-nitrogen to the total rest-

nitrogen is (in % of the latter) for leucosin 69.2, for gliadin 55.6, and for glutenin 31.7 (mean for all pH values).

TABLE II

ACTION OF YEAST PROTEOLYTIC ENZYMES ON THE INDIVIDUAL PROTEINS
(*in milligrams of nitrogen*)

pH	Amino-N			Rest-N		
	Beginning	End	Increase	Beginning	End	Increase
			Leucosin			
3.2	2.15	5.25	3.10	9.10	14.30	5.20
3.7	2.57	7.50	4.93	9.05	15.80	6.75
4.7	2.20	7.10	4.90	8.90	15.99	7.09
4.9	2.10	6.90	4.80	9.00	15.40	6.40
5.3	1.98	6.60	4.62	9.10	15.30	6.20
5.6	1.98	6.20	4.22	9.00	15.00	6.00
6.2	2.00	4.65	2.65	8.80	13.40	4.60
			Gliadin			
3.2	1.20	3.99	2.79	7.20	11.00	3.80
3.7	1.40	5.62	4.22	9.25	15.80	6.55
4.7	1.20	4.90	3.70	8.00	14.40	6.40
4.9	1.17	3.94	2.77	6.90	12.30	5.40
5.3	1.04	4.10	3.06	5.72	10.70	4.98
5.6	0.99	2.30	1.31	5.38	9.20	3.82
6.2	0.75	1.23	0.48	4.70	6.75	2.05
			Glutenin			
3.2	0.80	1.10	0.30	4.00	5.00	1.00
3.7	0.99	1.43	0.44	4.80	5.72	0.92
4.7	1.00	1.65	0.65	5.50	7.30	1.80
4.9	1.05	1.87	0.82	5.40	8.30	2.90
5.3	1.00	1.86	0.86	5.10	8.00	2.90
5.6	0.94	1.68	0.74	5.30	7.70	2.40
6.2	0.78	1.00	0.22	4.30	5.20	0.90

It is clear from these data that the specificity of the splitting of wheat proteins by the flour and yeast ferment mixtures is based on the specific properties of single proteins rather than on the specificity of flour and yeast proteolytic enzymes. But the control experiments with pure flour and yeast proteinases provided evidence that the apparent simplicity of this relationship is illusory.

For these latter experiments we prepared glycerol solutions of yeast and wheat flour proteinases by the method proposed by Grassmann and Dyckerhoff (1928) for the isolation of yeast proteinase. The proteinase preparations after purification by adsorption on alumina $C\gamma$ and corresponding elution were activated by H_2S and mixed with equal amounts of glycerol. The splitting of gliadin and glutenin by these proteinases was investigated. The results are given in Table III.

The action of the pure proteinases of flour and yeast on gliadin

TABLE III

THE ACTION OF FLOUR AND YEAST PROTEINASES ON GLIADIN AND GLUTENIN
(in milligrams of nitrogen)

pH	Amino-N			Rest-N		
	Beginning	End	Increase	Beginning	End	Increase
			Gliadin (Flour proteinase)			
3.3	1.21	1.22	0.01	7.00	7.60	0.60
4.9	1.10	1.27	0.17	6.60	11.60	5.00
5.0	1.10	1.28	0.18	6.62	11.72	5.10
5.3	1.10	1.17	0.07	6.00	9.10	3.10
6.2	0.77	0.80	0.03	4.40	5.65	1.25
			Glutenin (Flour proteinase)			
3.3	0.97	0.97	0.00	5.00	5.40	0.40
4.9	0.96	0.98	0.02	5.36	9.10	3.74
5.0	0.95	0.95	0.00	5.30	9.15	3.85
5.3	0.88	0.95	0.07	5.30	7.50	2.20
6.2	0.83	0.83	0.00	5.30	6.00	0.70
			Gliadin (Yeast proteinase)			
3.3	1.10	1.30	0.20	7.00	12.90	5.90
4.9	1.10	1.30	0.20	7.18	12.93	5.75
5.0	0.77	0.81	0.04	6.40	10.70	4.30
5.3	1.10	1.19	0.08	4.40	6.60	2.00
6.2	1.20	1.23	0.03	7.20	8.00	0.80
			Glutenin (Yeast proteinase)			
3.3	0.90	0.93	0.03	5.56	10.50	4.94
4.9	0.89	0.98	0.09	5.57	10.76	5.19
5.0	0.88	0.97	0.09	5.38	8.80	3.42
5.3	0.81	0.85	0.04	5.30	7.00	1.70
6.2	0.97	0.99	0.02	5.00	5.60	0.60

and glutenin differs from the action of the enzyme mixtures of flour and yeast extracts. The increase in the nitrogen of the free amino-groups is insignificant, as is seen from the following values in which the increase in amino nitrogen is expressed as per cent of the increase in nitrogen not precipitated by trichloracetic acid:

Protein	Enzyme	
	Flour	Yeast
Gliadin, %	3.0	3.0
Glutenin, %	<1.0	1.6

These relations allow the conclusion that the first stage in the splitting of proteins is the process of disaggregation of protein molecules into simpler polypeptide chains without a production of free amino- and carboxyl groups.[1]

[1] Balls, A. K., and Hale, W. S. (Cereal Chem. 13: 54–60 (1936)), conclude also that "during the process of dough fermentation the action of a proteinase in flour changes the (colloidal) properties of the wheat proteins."

The optimal pH values for flour proteinase were found to be 4.9 to 5.0, both for gliadin and also for glutenin. For yeast proteinase the pH values were 3.3 to 4.9. In the case of purified proteinases, we find consequently that the specificity of the enzyme predominates, while the specificity of the proteins finds its expression only in the relative rates of their splitting. The relation of the rate of gliadin breakdown to that of glutenin is for flour proteinase 1.38 : 1.00 and for yeast proteinase 1.18 : 1.00.

Summary

The optimum pH for the action of the natural mixture of proteolytic enzymes of wheat flour extracts on the proteins of wheat gluten are, for leucosin, 3.7 to 4.7; for gliadin, 3.7; and for glutenin, 4.9 to 5.3.

The optimum pH for the action of the natural mixture of proteolytic enzymes of yeast extracts on leucosin is 3.7 to 4.7; on gliadin, 3.7; and on glutenin, 4.9 to 5.3.

The splitting of glutenin, *ceteris paribus*, is less than the splitting of leucosin and of gliadin.

The optimum pH values for the action of pure wheat flour proteinase on gliadin and glutenin are identical and equal to 4.9 to 5.0.

The optimum pH for the action of pure yeast proteinase on gliadin and glutenin is also the same and equals 3.3 to 4.9.

The action of pure flour and yeast proteinases is a disaggregating rather than a proteolytic one since it is accompanied only by insignificantly small increases in amino nitrogen.

Acknowledgment

We have to thank Mrs. E. D. Dombinski for her assistance in the analytical work and V. A. Nicolaieff for his interest in this investigation.

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THE EFFECT OF MILK SOLIDS ON FERMENTATION REACTIONS [1]

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(Read at the Annual Meeting, June 1936)

Even though milk solids are now a common ingredient in bread doughs, their effects upon yeast growth and enzyme activity have not been directly investigated. This paper will summarize briefly the results of certain researches that have been made intermittently in this field during the course of the work of the past 7 years. These results have been reported only to the American Dry Milk Institute, the sponsers of this research program. Some of the general conclusions that have been reached through this work have appeared in a bulletin published by this organization (see Brouilett *et al.*, 1935).

Effect on Proteolytic Activity

There have been many investigations of the possible effect of various dough ingredients upon the activity of the proteolytic enzymes. The authors (see Skovholt and Bailey, 1935) attempted to determine the effect of milk solids upon the rate of proteolysis in bread doughs. No effect was registered in these investigations but the inadequacy of available methods for studying the attacks that proteases may make on the protein complex cause such negative results to be more or less inconclusive.

Effect on Diastatic Activity

Many dried milks and flours have been used in investigations of the effect of milk solids upon diastatic activity in flour suspensions and doughs. The most complete study involving two flours was made several years ago using the Rumsey digestion procedure and the Quisumbing-Thomas method for the determination of reducing sugars. Averaged data only are given in Table I.

The two flours differed appreciably in diastatic activity but the depressing effect of milk solids upon sugar production is evident with both when used in unbuffered suspensions. This effect is increasingly pro-

[1] Published as paper No. 1430. Scientific Journal Series, Minnesota Agricultural Experiment Station.

TABLE I

HYDROGEN-ION CONCENTRATION AND AMOUNT OF REDUCING SUGAR PRODUCED IN FLOUR SUSPENSIONS

(Reducing sugar produced given as milligrams of maltose per 10 g. flour)

Suspension	Flour A				Flour B			
	pH	Reducing sugar			pH	Reducing sugar		
		1 hour digestion	2 hours digestion	3 hours digestion		1 hour digestion	2 hours digestion	3 hours digestion
Milk-free, unbuffered	5.90	94.0	115.6	133.5	6.04	134.5	184.2	222.8
6% dried skim-milk, unbuffered	6.49	67.5	83.7	90.8	6.48	81.0	99.7	112.6
Milk-free, buffered [1]	4.85	102.9	—	—	4.70	164.0	—	—
6% dried skim-milk, buffered	4.87	95.9	—	—	4.73	165.0	—	—

[1] Sorenson's citrate buffer used to elimate the effect of milk solids on hydrogen-ion concentration.

nounced with an extension of the digestion period. In suspensions buffered to the same hydrogen-ion concentration, the rate of sugar production is unchanged by the addition of milk solids.

The amount of sugar produced in these suspensions is graphically presented in Figure 1. Sugar production in suspensions buffered to the same hydrogen-ion concentration was determined only after a 1-hour digestion period.

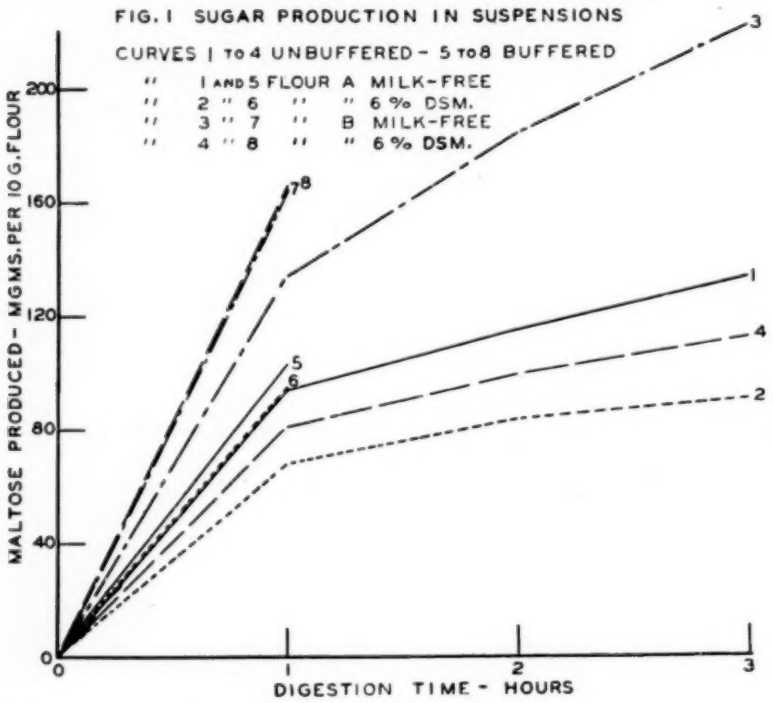
The depressing effect of milk solids upon diastatic activity also was eliminated by modifying the hydrogen-ion concentration of both flour suspensions and doughs by the addition of lactic acid in just sufficient amounts to overcome the acid-reducing effect of the milk solids. It seems evident that sugar-production is inhibited by milk solids only because of the effect upon the active acidity of the system.

Effect on the Rate of Gas Production in Doughs

In contrast to the depressing effect upon diastatic activity, there has been observed repeatedly an accelerating effect of milk solids upon the rate of gas production in fermenting doughs. Such an acceleration had been observed invariably when milk solids were introduced into doughs made with commercial flours and containing other basic dough ingredients in commonly-used concentrations.

A study was made of gas production rates when using flours of widely varying types, and diastatic activity in doughs containing varying

amounts of sugar or diastatic malt. Only flour, water, and 2% yeast were used in the preparation of dough except for the additional ingredients mentioned in the tabulation of data. Readings of gas pro-



duced were made at 15-minute intervals using a method essentially like that described by Bailey and Johnson (1924). Cumulative amounts of gas generated by doughs from 40 g. of flour, held at 28° C., are recorded for successive 30-minute periods in Table II. The recorded values for the diastatic activity of the flours used were determined by the Blish-Sandstedt method.

Some of the typical data from this table are graphically presented in Figures 2 and 3. The effects of the addition of dry skim-milk to doughs made with a commercial flour (D) are compared with results obtained with the low-diastatic, experimentally-milled flour (E), both with and without sugar as a dough ingredient.

When no sugar was introduced into the doughs prepared with commercially milled flours, a depressing effect of milk solids upon gas production was evident. Such results might be expected due to the dependance of the gas-producing enzymes upon diastase for the sugar needed. Results with the experimentally and semi-commercially milled

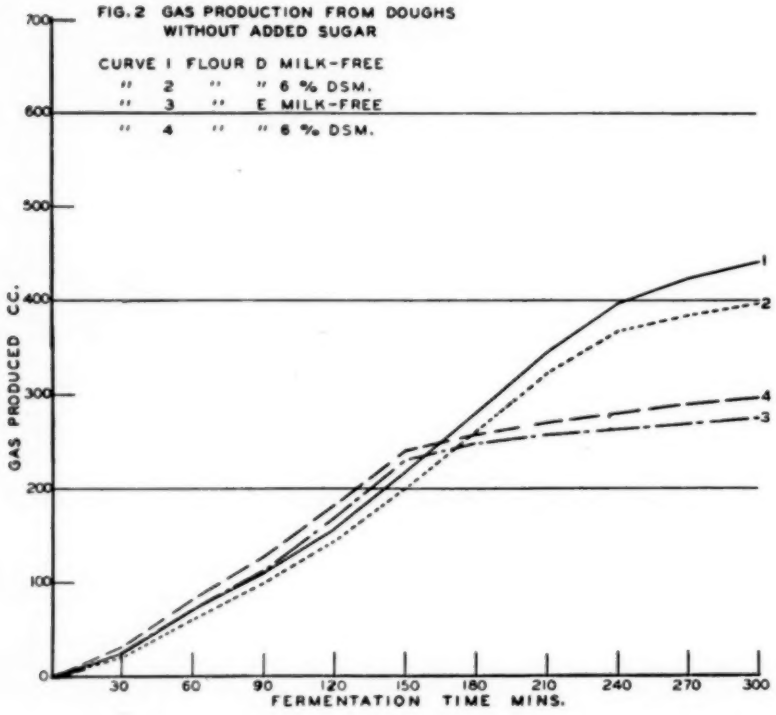
TABLE II

EFFECT OF DRY SKIM-MILK UPON GAS PRODUCTION IN DOUGHS OF VARIABLE COMPOSITION

(*Cubic centimeters of CO_2 for each time interval*)

Fermentation time, *minutes*	30	60	90	120	150	180	210	240	270	300
Flour C—Commercially milled, Diastatic activity 199, No sugar or malt										
Milk-free	28	73	115	167	225	293	358	409	439	
6% dried skim-milk	24	71	114	166	224	285	352	386	407	
Flour D—Commercially milled, Diastatic activity 194, No sugar or malt										
Milk-free	24	65	108	156	217	280	345	397	423	441
6% dried skim-milk	20	60	99	144	200	260	322	367	384	397
Flour E—Experimentally milled, Diastatic activity 122, No sugar or malt										
Milk-free	25	70	112	169	230	248	256	263	268	274
6% dried skim-milk	31	82	127	182	240	257	269	280	290	297
Flour F—Semi-commercially milled, Diastatic activity 143, No sugar or malt										
Milk-free	22	65	113	162	214	271	311	321	328	334
6% dried skim-milk	19	62	110	156	208	269	313	327	338	345
Flour G—Semi-commercially milled, Diastatic activity 222, No sugar or malt										
Milk-free	23	68	117	169	238	308	363	375	384	391
6% dried skim-milk	23	67	117	163	230	303	374	394	407	418
Flour C—With 5% sugar										
Milk-free	31	84	145	211	279	344	405	458	513	560
6% dried skim-milk	38	94	159	229	309	395	482	557	625	694
Flour D—With 5% sugar										
Milk-free	26	71	125	182	246	308	366	420	471	516
6% dried skim-milk	23	67	121	180	247	322	402	479	549	614
Flour E—With 5% sugar										
Milk-free	17	62	107	165	238	304	381	455	523	581
6% dried skim-milk	30	80	140	210	283	365	455	550	634	703
Flour C—With 0.5% diastatic malt (60°L.)										
Milk-free	28	75	122	178	242	312	384	452	514	553
6% dried skim-milk	31	79	125	179	244	319	395	472	522	546
Flour E—With 1.0% diastatic malt (60°L.)										
Milk-free	27	73	124	176	248	328	408	471	496	516
6% dried skim-milk	28	74	125	177	248	326	407	455	479	501

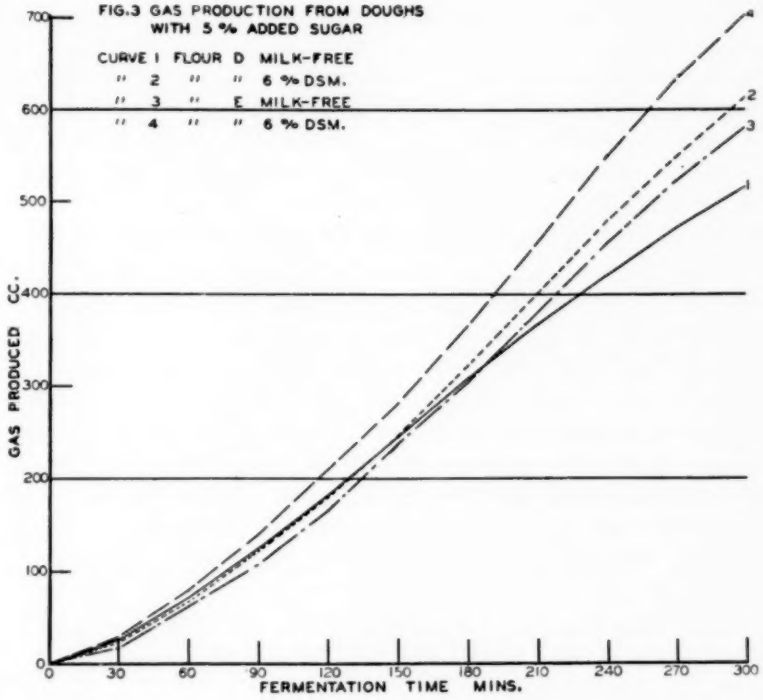
flours were surprising. The level of diastatic activity in these flours ranged from very low to moderately high. The actual amount of gas produced was proportional to the diastatic activity. The length of fermentation time that elapsed before a decided retardation in rate of gas



production became evident was also related to diastatic activity. With these flours, milk solids did not depress the rate of gas production, however, and there was a small accelerating effect during the latter part of the fermentation period. It might be expected that in such systems of limited diastatic activity the previously observed depressing effect of milk solids on this rate of sugar production would cause a reduction in fermentation rate as measured by gas evolution.

It is known that methods used in tempering and grinding wheat function in determining the apparent diastatic activity of the resulting flours. Such effects are probably upon starch susceptibility rather than upon concentration of diastase present. The controlling factor in determining rate of sugar production during the digestion of these flours may have been starch susceptibility. It is possible that in systems of this type the rate of sugar production is not reduced by the decreased

hydrogen-ion concentration caused by milk solids, or that some counteracting stimulation is effected by components present in milk. Such speculation needs the support of further evidence.



In all doughs containing liberal amounts of added sugar, the usual accelerating effect of milk solids upon the rate of gas production was in evidence. When diastatic malt was used with two of the flours, no significant effect of milk solids was registered. These results may have been due to a counteracting effect of two tendencies that already have been noted. Due to the addition of supplementary diastase, sugar may have been produced rapidly enough to largely avoid restricting the activity of the gas-producing enzymes, even though sugar-production may have been retarded, in the case of flour C at least, by the presence of milk solids. Counteracting any possible restriction of this type is the accelerating effect of milk solids that causes more rapid conversion of sugar into the gasses of fermentation.

Is the Effect on Yeast Growth or Enzyme Activity?

It is believed that several mechanisms are involved in fermentation by yeast organisms. No attempt will be made to review or even enu-

merate the many contributions that have been made to knowledge in this field. Disagreements still exist in the attempts made to formulate a complete theory of yeast fermentation. It seems evident, however, that an increased rate of gas production could be caused by (*a*) accelerating the growth and rate of reproduction of the yeast cells, thus creating more enzyme producers, or (*b*) by affecting the rate of production of enzymes and co-enzymes by the protoplasm of the living cells, or (*c*) by stimulating the activity of these enzymes. A fairly close relationship may exist between the first two factors mentioned although it is likely that enzyme elaboration is a different mechanism than that involved in yeast growth and reproduction.

There is some difference of opinion as to the amount of yeast growth that occurs in bread doughs. Several factors that may be involved in determining the amount of growth that may occur have recently been reviewed by Simpson (1936). According to Simpson, no yeast growth occurs in doughs that contain yeast in a concentration of about 2% or more of the flour weight used. Other factors may appreciably modify this limiting concentration, however, particularly the strain of yeast selected for study. It is possible that the accelerating effect of milk solids upon gas production may have been due to a stimulating effect upon yeast growth and reproduction in the systems investigated. It seemed desirable to determine if this were the case or if the effect was largely upon enzyme activity.

Several methods have been proposed for effecting fermentation reactions in the absence of the living yeast cell. Two general methods were used in this investigation in attempting to secure an active fermentation by the zymase complex but free from the complicating effect of possible yeast growth. One of these methods involved various procedures in attempting to effect a rupturing of the yeast cell by mechanical, desiccating or autolytic action. After such treatments one of these preparations was placed in doughs as an extract while others were used in a dried form. The second method that was tried involved the use of antiseptics that supposedly prevent yeast reproduction without seriously impairing the activity of the enzymes already present. These preparations from yeast were used in varying concentrations. The only other dough ingredients regularly used were flour, water, and 5% sugar, based on flour weight, to insure an adequacy of fermentable material. One of each pair of doughs also contained 6% dried skim-milk.

Most of the inactivation methods failed to give the desired results. An attempt was made to inactivate yeast by freezing at — 12° C., followed by a grinding of the yeast in a mortar at that temperature. This method, originally used by Buchner, has been followed by several in-

vestigators. It appeared that little inactivation had been effected in this preparation and the resulting gas production curves had the characteristics of those obtained with live yeast. Only one trial was given this method since relatively large amounts of yeast were needed and it appeared that other methods might give the desired results with less time consumed in preparation.

A method proposed by Lebedev (1911) for obtaining zymase extract from yeast is described in most reviews of the fermentation problem. Two preparations made by this method caused an almost imperceptible amount of fermentation as measured by gas production. Other investigators have reported variable success with this method since the activity of the preparation seems to depend largely upon the yeast selected for use.

A preparation known as zymin is produced from yeast by a preliminary drying followed by a treatment with acetone and ether. A method for preparing zymin suggested by Dann and Quastel (1928) was followed. The results with this preparation indicated that yeast reproduction had not been eliminated although the amount of gas production was less than with equivalent amounts of untreated yeast.

Myrbäck and V. Euler (1929) have reported that a suitable drying of yeast will almost completely eliminate whole yeast cells with relatively little loss in fermentative power. Such preparations also gave fermentation rates indicative of yeast growth although no attempt was made to verify this by actual count. Both zymin and the last mentioned type of dried yeast preparation were used in doughs without milk solids as well as in doughs containing 6% dried skim-milk. An appreciably increased rate of gas evolution was caused by the presence of the milk solids. This might be taken to indicate that milk solids exerted a stimulating effect upon the zymase complex though it does not prove that an effect on rate of yeast growth was not also in evidence.

Chloroform and toluene were used as yeast inactivators in several trials. Duchacek (1909) and other investigators have reported that the proper concentration of these compounds will prevent yeast reproduction with little effect on enzyme activity. Optimum concentrations as given for solutions may mean little when applied in bread doughs because of different liquid-solid proportions and because of possible adsorptions by the flour of the system. Toluene was used in concentrations of 5% and 10% of flour weight and chloroform only in the higher of these two concentrations. These concentrations are appreciably higher than those recommended when dealing with solution. Both fresh and dried yeast were used, the concentrations being 10% and 5% of flour weight, respectively. Different concentrations were used because of the greatly

lowered moisture content of the dried preparations. The yeast was stirred into the water required for dough preparation followed by the addition of the chloroform or toluene, then allowing a period of several minutes before addition to the other dough ingredients. First readings of gas production were taken at intervals of 1 hour or less. After 5 hours the collecting burettes were reset and the evolved gas was allowed to accumulate overnight followed by a reading of total evolution after 22 hours of fermentation. Some of the averaged values giving cumulative gas production in cubic centimeters from doughs containing 40 g. of flour are found in Table III.

TABLE III

GAS PRODUCTION IN DOUGHS CONTAINING TREATED YEASTS

(*Cubic centimeters of CO_2 per time interval*)

Fermentation time, *hours*	1	2	3	4	5	22
Untreated, dried yeast, milk-free	43	98	169	267	394	—
5% toluene, dried yeast, milk-free	14	19	23	25	27	39
5% toluene, dried yeast, 6% dry skim-milk	15	21	26	30	33	48
5% toluene, fresh yeast, milk-free	23	54	74	84	89	95
5% toluene, fresh yeast, 6% dry skim-milk	15	39	67	87	104	139
10% toluene, fresh yeast, milk-free	23	47	62	69	72	80
10% toluene, fresh yeast, 6% dry skim-milk	17	36	60	81	97	122
10% chloroform, dried yeast, milk-free	28	38	44	48	—	61
10% chloroform, dried yeast, 6% dry skim-milk	23	31	36	39	—	65
10% chloroform, fresh yeast, milk-free	19	24	29	30	—	40
10% chloroform, fresh yeast, 6% dry skim-milk	19	25	30	32	—	48

The fermentation rate obtained when using untreated dried yeast (first line, Table III) shows the acceleration during the entire 5-hour fermentation period that appears to be typical when active yeast and sufficient sugar are present. Both toluene and chloroform treatments eliminated this tendency completely except with the toluene-treated fresh yeast in which cases a slight acceleration in fermentation rate is shown during the first 2 hours. This may have been due to incomplete elimination of yeast growth at the beginning of the test. All other fermentation rates are those believed to be typical of enzyme reactions.

The point of chief interest appears upon direct comparison of the dough pairs, one prepared with and the other without milk solids. Averaging all such pairs, it was found that milk-free doughs produced 63 cc. and those containing 6% dried skim-milk evolved 86 cc. of gas during the 22-hour period. If the two pairs of doughs containing toluene-

treated fresh yeast are omitted, these values are 47 cc. and 54 cc. respectively. The averaged increased gas production due to dried skim-milk is thus about 33% when comparing all pairs and about 15% when including only those doughs that give every indication of having a fermentation rate typical of normal enzyme reactions. These values compare with an averaged total gas production increase due to 6% dried skim-milk of 21% from normally fermenting doughs containing an adequate amount of sugar and 2% active yeast. Such comparisons cannot be accepted as proof that dried skim-milk does not stimulate yeast growth but it does indicate that the observed effects are probably largely due to an activation of the zymase complex. It seemed desirable to determine what rôle the reduction in hydrogen-ion concentration caused by milk solids might play in this increased activity.

Effect of Hydrogen-Ion Concentration upon Zymase Activity

Two of the experiments that were conducted with doughs of varying hydrogen-ion concentration gave some interesting information on the activity of zymase in such systems. These doughs were prepared with flour, water, 5% sugar and 10% fresh yeast treated with toluene in a concentration of 7% of flour weight or about 10% of the weight of water used in dough preparation. Five of the doughs studied were prepared without the addition of milk solids while three of them contained 6% dried skim-milk. Additions of lactic acid and in one case, of sodium hydroxide, effected the desired range in hydrogen-ion concentration in the doughs. Determinations of pH were made after 3 hours of fermentation upon aliquots of the same doughs as used in gas production studies. A few determinations that were made directly after mixing of the doughs showed that these systems were changing very slowly in hydrogen-ion concentration due to the low fermentation rate. In one experiment, gas production readings were made frequently during the first 5 hours, and again after 8 and 22 hours. The reading after 8 hours of fermentation was not obtained in a second series. All values given in Table IV are averages of duplicate gas production determinations from doughs made with 40 g. of flour, held at 28° C.

Two effects are evident in the data of Table IV. One is the retarding effect of increased hydrogen-ion concentration upon zymase activity. This is evident in both the series of doughs with milk solids and in those not containing milk. The first three lines of data show the reduced activity in doughs containing milk when increments of lactic acid were added. The data from the second experiment show the increasing effect of sodium hydroxide and the decrease due to lactic acid in the total gas produced from milk-free doughs. The differences in gas production

and in hydrogen-ion concentration are both relatively small. It seems evident that this is an effect of hydrogen-ion concentration upon zymase since yeast growth was evidently eliminated and since both sodium and lactate ions were involved in addition to hydrogen and hydroxyl ions. These results are not in agreement with some others that have been reported although it is recognized that factors such as nutrient medium and temperature are important in determining the optimum pH for zymase activity.

TABLE IV

GAS PRODUCTION IN DOUGHS OF VARIOUS HYDROGEN-ION CONCENTRATION CONTAINING TOLUENE-TREATED YEASTS

(*Cubic centimeters of CO_2 per time interval*)

Time of fermentation, *hours*	1	2	3	4	5	8	22	pH
Experiment 1								
6% dry skim-milk	18	39	65	88	106	145	—	6.12
6% dry skim-milk, 5.2 cc. 0.1 N lactic acid	17	38	63	85	104	142	—	5.87
6% dry skim-milk, 10.4 cc. 0.1 N lactic acid	17	34	57	76	92	130	—	5.70
Milk-free	24	51	68	78	83	89	100	5.85
Experiment 2								
Milk-free, 2.6 cc. 0.1 N NaOH	25	56	75	84	87	—	100	5.98
Milk-free	24	52	69	77	80	—	94	5.80
Milk-free, 2.6 cc. 0.1 N lactic acid	25	52	66	74	77	—	91	5.64
Milk-free, 5.2 cc. 0.1 N lactic acid	25	47	61	67	71	—	86	5.39

Only a part of the zymase activating effect can be attributed to the reduction in hydrogen-ion concentration caused by milk solids. In comparing gas production rates from doughs with and without milk solids it is evident that the milk-free doughs generate gas more rapidly than those containing milk during the early stages of fermentation. An accelerating effect of milk solids soon becomes evident and at the end of about 3 to 4 hours the gas production is in an inverse order to hydrogen-ion concentration when comparing doughs of both types. The accelerating effect of milk solids continues, however, and after 8 hours of fermentation such doughs have generated considerably more gas than those without milk regardless of hydrogen-ion concentration. This stimulation of activity continued to be so pronounced that the amount of gas generated overnight from the milk doughs exceeded the capacity of the burettes, meaning that more than 50 cc. had been evolved in the period between the 8 and the 22-hour readings. A similar difference in type of fermentation curve from doughs with and without milk solids was observed when toluene-treated fresh yeast was used. obtaining the data reported in Table III.

The exact nature of this peculiar effect of milk solids upon fer-

mentation rate, when using certain treated yeasts, could only be determined after extended investigation. The investigation of this phase of the problem just reported has established only the probability that milk solids activate the zymase complex in doughs partly because of an effected reduction in hydrogen-ion concentration. It has also been shown that milk solids have other effects upon this complex that are activating in nature when considering extended fermentation periods. The use of other types of yeast-free zymase preparations would be highly desirable in obtaining additional evidence.

Summary

Proteolytic activity in doughs is not affected by milk solids as judged by applications previously made of available methods though it is believed that such methods may not measure adequately all types of attacks that proteases may make on the protein complex.

Diastatic activity in doughs is retarded by the introduction of milk solids due to the effected reduction in hydrogen-ion concentration.

Gas production from fermenting doughs is accelerated by milk solids if sufficient sugar is available. From sugar-deficient doughs made with ordinary commercial flours, milk solids effect a reduction in amount of gas produced due to a retarding effect on diastasis. With certain experimentally milled flours this retarding effect is not in evidence even in systems low in diastatic activity. Such low diastatic activity may be due to a low starch susceptability to diastase attack not adversely affected by addition of milk solids.

Yeast treated to inhibit growth was used in doughs and results seemed to indicate that milk solids increase the activity of the zymase complex.

Activation of zymase by milk solids appeared to be partially due to an effected reduction in hydrogen-ion concentration. Other stimulating effects of milk solids upon fermentation by zymase from yeast were also noted.

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SOME OBSERVATIONS ON METHODS OF ASHING CEREAL PRODUCTS [1]

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(Read at the Annual Meeting, June 1936)

Cereal chemists have sought for years to find accurate methods that could be used in the ashing of cereal products so as to obtain consistent reproducible ash values within a comparatively short time. If too low a temperature is used the charred mass remains black, even after hours of heating, with little, if any, apparent change. If, on the other hand, the temperature is too high, the ash material fuses into a glass-like mass which is liable to contain occluded carbon. It is well known that certain of the more volatile mineral constituents are lost, even though the ashing is done at a relatively low temperature.

In order to hasten the process of ashing and at the same time prevent fusion of the ash, several methods have been proposed.

Goldtrap (1917) states that it is essential for the sample to be ashed at a low temperature, not above a dull red heat, that an apparent loss in ash is due to the fact that the phosphates are "reduced" to metaphosphates, which, however, by adding nitric acid are again "oxidized" to orthophosphates.

Hertwig and Bailey (1924) suggested moistening flour with equal parts of glycerine and alcohol, before burning off the carbon.

Coleman and Christie (1925) pointed out that "fusion of flour ash causes high and irregular results."

Tausz and Rumm (1925) wrapped the flour in gold foil which was placed in a Rose crucible. After coking, oxygen was introduced.

Goske (1925) used finely powdered, calcined pumice to prevent the formation of a compact mass of coke.

Mangels (1925) stated that the addition of calcium acetate makes it possible to ignite at a higher temperature without volatilization of the ash constituents.

[1] Food Research Division Contribution No. 286.

Brendel (1926) modified the calcium-acetate method. He first charred the flour, then allowed the cinder to cool, after which it was moistened with the calcium-acetate solution, dried, replaced in the furnace and burned in a stream of oxygen.

Micka (1927) compared results obtained by ashing at different temperatures, using different kinds of ashing dishes, and different aids. He concluded that platinum dishes are the most suitable for ashing flour; that fusion does not lead to irregular results if platinum dishes are used; that alcohol-glycerine reagent and alundum do not shorten the burning time at high temperatures; that results by three modifications of the calcium acetate method were unreliable unless the temperature was above 900° C.; and that the best method for ashing flour proved to be the direct burning at high temperature in platinum without the addition of other substances.

Sullivan and Near (1927) state that the A.O.A.C. method does not give accurate results with some of the Northwestern wheat flours, since a higher temperature than 550° C. is necessary to obtain a carbon free ash. Temperatures above 630° C. cause the ash to fuse and irregular results are obtained. They char the sample at 400° C., then incinerate at 620° C. for 14 to 16 hours.

Johnson and Scott (1928) found that a fluffy ash was correlated with a high potassium and a relatively low phosphorus content, the reverse being true in the case of a fused ash. When the incineration time is 16 hours and the temperature 485° to 725° C. the percentages of ash obtained agreed closely in replicates, but that when the incineration was conducted at 750° C. the percentages of ash appeared to be low.

Spalding (1930) described a method which is an outgrowth of the calcium-acetate-oxygen method proposed by Brendel (1926). Spalding substituted magnesium acetate for the calcium acetate. Only about 15 minutes are required to obtain the ash from 3 g. of flour by this method.

Walters (1930) used the salts of the rare earth metals, lanthanum, cerium, yttrium, and thorium, all in the form of nitrates dissolved in 40% alcohol. The concentrations of the solutions were such that 10 cc. of each of them would contain from 5 to 20 mg. of the respective oxide after incineration. With these aids he was able to reduce the time of ashing 5 g. of flour from about 5 hours to about 25 minutes or possibly less. With the aid of oxygen Walters succeeded in ashing 5 g. of flour with 20 mg. lanthanum oxide in 5½ minutes, of which 4½ minutes were used in driving off the volatile matter. Walters recommended using approximately 7½ mg. of lanthanum oxide when ashing 5 g. of soft wheat flour and 15 mg. when ashing the same quantity of hard wheat flour.

Radeff (1931) gives a method for determining flour ash which uses a combination of the rare earth metal nitrates with alcohol and glycerine. In this method the lanthanum or other nitrate is dissolved in a solution containing 40% alcohol, 40% glycerine, and 20% water. Sufficient of the respective nitrates are used so that 10 cc. will yield approximately 10 mg. of oxide.

Potts (1931) used two muffle furnaces, one kept at 800° F. in which he allowed the samples to remain 40 to 50 minutes, after which he transferred them to another furnace kept at 1100° F. Into this furnace he conducted a stream of oxygen at the rate of 150 bubbles per minute. The ash samples were left in the second furnace for ¾ to 1½ hours, or until they assumed a white or light gray color.

Bowen (1932) first charred his sample in a furnace with the door open. After charring he removed the sample, allowed it to cool and then added dilute nitric acid, dried and again heated at a dull red. The resulting ash was said to be perfectly white.

Davis (1932) conducted collaborative tests with the magnesium-acetate-alcohol method in comparison with the collaborators' regular method. Twenty-five collaborators reported on five flour samples. There was a slightly higher relative variability in the magnesium-acetate-alcohol results than in the results by the collaborators' regular method, but a large majority of the collaborators were of the opinion that the proposed quick ash method was sufficiently dependable for control work.

Working and Anderson (1934) studied "Quick Ashing Methods" and proposed a micro-method. They worked with 0.3 g. of flour moistened with a 70% alcoholic solution of magnesium nitrate. They used a flat platinum dish, with furnace temperature of 550° C. Time required, 5 to 10 minutes.

The official ash method of the Association of Official Agricultural Chemists (1930) for cereal foods specifies, "incinerate in a furnace at approximately 550° (dull red) until a light gray ash results or until no further loss in weight occurs."

The American Association of Cereal Chemists (1935) have adopted four methods for determining ash in flour and semolina: (*a*) The A.O.A.C. official method, (*b*) Direct-weighing method, (*c*) Glycerol-alcohol method, and (*d*) Magnesium-acetate method.

With the A.O.A.C. method the sample is incinerated at approximately 550° C. In the case of the direct-weighing method the sample is started at 450° C. and the temperature is gradually increased to 550° C. and held at this temperature until a light gray ash results. This requires 5 to 6 hours. The ash is then transferred to the balance pan and weighed. With the glycerol-alcohol method the alcohol is

ignited before placing the sample in the furnace which is held at about 575° C. until the ash becomes fluffy and gray-white. With the magnesium-acetate method the muffle furnace is held at 850° C. and the time required after the flaming has ceased and the furnace door closed is only 30 to 45 minutes.

In addition to the above described methods, glacial acetic acid and hydrogen peroxide have been proposed as ashing aids.

Experimental

Since the official A.O.A.C. ashing-method requires so much time for its completion, and so many rapid methods have been proposed, it was decided to study the merits of some of the more rapid methods. The two methods that seemed to have special advantages over the others were given first consideration.

The method of Walters (1930), which uses the nitrates of the rare earth metals as aids, was studied with bran, and wheat and rye flours of different grades; the nitrates of lanthanum, cerium, thorium, and yttrium being employed for this study. A sufficient quantity of each of these nitrates was dissolved in 40% alcohol so that 10 cc. of the solution would, upon ignition, yield approximately 15 mg. of oxide. Five-gram samples of hard and soft wheat flours were moistened with 5 and 10 cc. of lanthanum nitrate, respectively. The alcohol was burned off and the samples placed in a furnace held at 550° C. Approximately 3½ hours was required to complete the ashing operation. Similar results were obtained whether a 5 cc. or a 10 cc. portion of the solutions was used. The next step was to use an ashing temperature of 850° C., the same as recommended in the magnesium-acetate method. (Walters specified a bright cherry red.) Heating at this temperature (850° C.) and using solutions of cerium, thorium, and yttrium nitrates, a satisfactory light gray ash was produced with both hard and soft wheat flours in from ½ to ¾ hour.

Then a combination of the Walters method with the alcohol-glycerine method (see Radeff, 1931) was tried. The temperature of the furnace was reduced to 775° C. When the alcohol was ignited, or evaporated on a steam bath before placing the samples in the furnace, there was a tendency for more or less spattering at that high temperature. Both platinum and porcelain dishes were used and about equally good results were obtained with each. Duplicate results were secured with the thorium nitrate in a 40% alcohol solution, and in a 40% alcohol—40% glycerine—20% water solution. There seemed to be no advantage in the use of glycerine and more time was required in burning it off. Just as satisfactory ash results were obtained with a 3-g. sample as with a 5-g. sample and less time was required to get a white ash.

Three-gram samples of a patent flour, a clear flour, a white rye flour, and a dark rye flour were ashed at the same time at 850° C. with the aid of 10 cc. thorium-alcohol-glycerine solution. Closely agreeing duplicates were obtained in ¾ hour. When the temperature of the furnace was held at 750° C. and 10 cc. of the thorium-nitrate solution was used, it required 1 hour to ash a 3-g. sample of patent flour and 1½ hours to ash a 5-g. sample. At 800° C., using 10 cc. of the same strength solution of thorium nitrate with each 3-g. sample, 1 hour was required to satisfactorily ash white and light rye flours, while 1¼ hours was necessary for medium rye flour.

When the dark rye flour was ashed under the same conditions, the ash fused and contained occluded carbon. Satisfactory results were not obtained. To overcome this difficulty, the concentration of the thorium nitrate was increased. Solutions were made containing two, three, and four times as much of the thorium nitrate as in the first solution, the percentage of alcohol and glycerine remaining the same.

With double the quantity of thorium nitrate, the ash of dark rye flours was of somewhat better appearance, but still there was some fusion. With three and four times the quantity of thorium nitrate there was no fusion. The ash was light and fluffy but the blank was so large that it was considered objectionable.

Wheat bran was ashed using 40% alcoholic solutions of the different rare metal nitrates. The samples were first heated for 2 hours at 650° C. and then for 1½ hours at 700° C. with results as shown in Table I.

TABLE I

Ash Results on Wheat Bran Using Different Rare Metal Nitrates

Aid used	Ash	Character of ash
	%	
None	6.89	Slightly fused, dark
Lanthanum nitrate	6.94	Not fused, dark specks
Cerium nitrate	6.84	Not fused, slightly gray
Thorium nitrate	6.94	Fused, dark specks
Yttrium nitrate	6.88	Not fused, slightly gray

Wheat bran was then ashed without any aid and with the 40% thorium-nitrate-alcohol-glycerine solution. When the temperature was kept at 550° C. neither ash was satisfactory. When heated to 800° C. the ash was unsatisfactory as it fused and carbon was occluded. Increasing the quantity of thorium nitrate even up to four times the regular amount, did not prevent the fusion of bran ash at a temperature of 800° C.

When ashed at 700° C. for 1½ hours bran with three times the regular quantity of thorium nitrate in alcohol-glycerine solution,

yielded 6.96% of ash which was not fused, but contained some dark specks. The same type of ash was obtained when using four times the regular quantity of thorium nitrate. In this case the yield of ash was 7.09%.

Another ashing aid was prepared by adding 15 g. of anhydrous magnesium acetate to 1 liter of 95% alcohol, allowing it to stand overnight and filtering off the excess. Ten cubic centimeters of this solution gave a blank ash determination of approximately 34 mg. A comparison was made between this aid and the use of thorium-alcohol-glycerine solution with 2 g. samples of bran. With four times the regular concentration of thorium nitrate in the alcohol-glycerine mixture, the yield, when ashed at 700° C. for 1 hour, was 7.17%, and a similar sample ashed at the same temperature with 10 cc. of magnesium acetate yielded 7.12%. The ash in both cases was satisfactory.

Other simultaneous tests were made with wheat bran, wheat flour, and dark rye flour. In each case the results with the magnesium-acetate procedure duplicated those obtained with four times the regular strength of thorium nitrate in alcohol-glycerine mixture.

Samples of wheat bran, wheat flour, dark and white rye flours were ashed according to the A.A.C.C. method with the magnesium-acetate solution at 850° C., the temperature recommended in this method. When using 10 cc. of the acetate solution the wheat flour and white rye bran were completely ashed in 20 minutes, and the dark rye flour and wheat bran in 40 minutes. The bran ash, however, was fused. When 20 cc. of the acetate solution was used there was some fusion at this high temperature. The percentages of ash on these samples, however, duplicated closely those made by the A.O.A.C. method at 550° C. without any aid.

Two-gram samples of bran were ashed at 700° C. with 10 cc. of the following aids: alcohol and glycerine, thorium nitrate in alcohol, thorium nitrate in alcohol-glycerine, cerium nitrate in alcohol, cerium nitrate in alcohol-glycerine, and 3 cc. of the magnesium-acetate-alcohol solution. All of the ash samples were fused within ¾ hour except the one with the addition of cerium nitrate and the one with magnesium acetate. The ashing process was practically completed in 1½ hours, although some of the residues were very dark. The percentage of ash by the different methods was practically the same.

Since there was no fusion of the ash when cerium nitrate and magnesium acetate were used, comparative ashings were made with wheat bran, dark rye and wheat flours, using cerium nitrate in 40% alcohol and magnesium acetate in 95% alcohol, respectively, at temperatures of 850° C. and 700° C. The results are given in Table II.

At 550° C. a 2-g. sample of bran with magnesium acetate was

fully ashed in 5½ hours, while similar samples with cerium and thorium nitrates were not completed in 8 hours.

TABLE II

COMPARATIVE ASH RESULTS WHEN USING SOLUTIONS OF CERIUM NITRATE AND MAGNESIUM ACETATE

Material		Aid used		Blank	Temperature	Time required	Ash	Character of ash
Kind	Amount	Amount	Kind					
	Grams	*Cc.*		*Mg.*	*° C.*	*Hours*	*%*	
Wheat bran	2	15	$Ce_2(NO_3)_6$	23.4	850	¾	7.04	Fused, dark
Wheat bran	2	5	$Mg(C_2H_3O_2)_2$	19.3	850	¾	6.94	Fused, light gray
Dark rye flour	3	10	$Ce_2(NO_3)_6$	15.6	850	½	2.03	Not fused, light gray
Dark rye flour	3	5	$Mg(C_2H_3O_2)_2$	19.3	850	½	2.00	Not fused, light gray
Patent flour	3	10	$Ce_2(NO_3)_6$	15.6	850	½	0.42	Not fused, white
Patent flour	3	3	$Mg(C_2H_3O_2)_2$	11.6	850	½	0.42	Not fused, white
Wheat bran	2	10	$Ce_2(NO_3)_6$	15.6	700	2½	7.10	Not fused, gray
Wheat bran	2	3	$Mg(C_2H_3O_2)_2$	11.6	700	2½	7.10	Not fused, gray
Dark rye flour	3	10	$Ce_2(NO_3)_6$	15.6	700	1½	2.09	Not fused, light gray
Dark rye flour	3	3	$Mg(C_2H_3O_2)_2$	11.6	700	1½	2.07	Not fused, light gray
Patent flour	3	10	$Ce_2(NO_3)_6$	15.6	700	1	0.43	Not fused, white
Patent flour	3	3	$Mg(C_2H_3O_2)_2$	11.6	700	1	0.43	Not fused, white

Dark rye flour was completely ashed in 1½ hours at 550° C. without any aid or when using cerium nitrate and magnesium acetate. Thus, the addition of ashing aids to dark rye flour that is to be ashed at either 550° or 700° C. is of no advantage. The same results were obtained in 1½ hours whether cerium nitrate, magnesium acetate, or nothing was used. Wheat flour without aid was ashed in 4½ hours, while with either cerium nitrate or magnesium acetate ashing took place in 2½ hours. When ashed alone, at 700° C., wheat flour ash fuses. Wheat flour ash also fuses slightly when 5 cc. of cerium-nitrate solution is used. This, however, is not the case when 10 cc. of cerium-nitrate solution is used. With 3 cc. of the alcoholic-magnesium-acetate solution (which is sufficient to moisten the flour) there is no fusion and the ashing is completed in about ¾ hour. With this small quantity of liquid (3 cc.) no evaporation or preliminary treatment is necessary. The sample does not need stirring and the dish may be placed in the furnace within a minute after adding the solution.

The blank from this magnesium-acetate solution is, however, so large in proportion to the ash from 3 g. of patent flour that it was decided to use a more dilute solution. It was found that 6 g. of the anhydrous magnesium acetate in 1 liter of 95% alcohol gave a blank of 5 mg. of MgO from 3 cc. of the solution. Thus, the magnesium-acetate method has been modified by lowering the temperature and also by reducing the blank.

At 700° C., in from ½ to ¾ hour, using 3 cc. of this magnesium-acetate solution (blank, 5 mg. MgO), with 3 g. of patent flour, a white porous ash was obtained which duplicated the results secured by

using the official A.O.A.C. method. This modified magnesium-acetate method is the most satisfactory rapid method so far tested by the writer for ashing wheat flour. For ashing a 2 g.-sample of wheat bran, three times as much of the magnesium-acetate solution was used as for flour and the time of ashing was doubled. Results are shown in Table III.

TABLE III

COMPARISON OF ASH RESULTS BY A.O.A.C. AND THE MODIFIED MAGNESIUM-ACETATE METHODS

Material	Method	
	A.O.A.C. (550° C.—10 hours)	Modified $Mg(C_2H_3O_2)_2$ (700° C.—¾ hour)
	% Ash	*% Ash*
Wheat bran	7.15 (dark)	7.08[1] (gray)
	7.18 (dark)	7.09[1] (gray)
White rye flour	0.72	0.69
	0.70	0.70
Patent flour	0.42	0.42
	0.42	0.42

[1] With the bran (2 g.) three times the regular quantity of magnesium acetate was used and the ashing was continued for 1½ hours.

Summary

A review of the literature regarding methods for ashing cereal products, with special reference to rapid methods for ashing flour, is given. A study was made of the conditions most favorable for ashing wheat flour, wheat bran, and rye flours of different grades.

The Walters method of using the nitrates of lanthanum, cerium, thorium, and yttrium was studied, also, a combination of this method with the alcohol-glycerine method. The magnesium-acetate method of the American Association of Cereal Chemists was also compared with the official method of the Association of Official Agricultural Chemists, and with Walters' rare-elements method with modifications.

The method found most satisfactory was a modification of the magnesium-acetate method. When 3 g. of flour (either wheat or rye) is moistened with 3 cc. of an alcoholic solution of magnesium acetate equivalent to a blank of 5 mg. MgO (6 g. of the anhydrous salt per liter), and incinerated in a muffle furnace at 700° C., a white fluffy ash is obtained in approximately ¾ hour. In the case of bran, using a 2-g. sample, 3 times the amount of magnesium-acetate solution must be used and ashing continued for 1½ hours. The results duplicate those of the official A.O.A.C. method.

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DETECTION OF SULFUR DIOXIDE IN FLOUR

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(Received for publication October 19, 1936)

Sulfur dioxide frequently is responsible for complaints on the baking quality of flour and a simple method for confirming its presence should be of value. The usual manner in which flour comes in contact with this gas is through the fumigation of warehouses by the burning of sulfur and the resultant sulfur dioxide is absorbed and retained by the flour.

Its presence is usually first suspected when mixing the dough; it having a soft, inelastic character much resembling a slightly stiff cake batter. This tightens up considerably as fermentation proceeds; however, the resultant loaf will be injured or ruined to an extent depending on the amount of sulfur dioxide absorbed.

The viscosity of a flour so injured will be greatly decreased. A flour which would show definite softness on mixing may possibly produce a passable loaf, yet the viscosity of the flour might only be 50% of its normal value and, as a rule, much less.

Confirming Presence of Sulfur Dioxide

To confirm the presence of sulfur dioxide, slick the suspected sample along side of a standard, which is known to be free from this gas, just as in the Pekar or slick test. Instead of using water, dip the slick in a potassium iodide—starch solution. The concentration of iodide and starch is not important; a gram of iodide and a half-gram of boiled starch to a beaker of water are satisfactory. This wetted slick is now passed carefully through air containing a light concentration of chlorine gas and the liberated iodine will give a light purple coloration to the slick. Care must be exercised not to over-treat with chlorine gas as in such case too much iodine will be set free for the quantity of sulfur dioxide present. It should not be found difficult to secure a light coloration with proper care. Within a few minutes the sulfur dioxide, if present, will have diffused to the iodine and reduced it to the colorless iodide. The standard will remain colored, whereas the sulfur dioxide contaminated sample will fade out to a normal color. If sulfur dioxide was present, the contrast on the slick will be very marked.

Agitator Accummulation

An occasional complaint sample will give a brown color, usually spotted, when first dipped in the potassium iodide-starch solution. This can usually be attributed to accumulated material which has collected in a bleaching agitator and later becoming free and dropping into the flour stream. This material will seriously interfere with the baking quality of flour but the dough character and viscosity are not affected as with sulfur dioxide contaminated flour.

THE SELENIUM AND CYSTINE CONTENT OF SOME PARTIAL HYDROLYSIS PRODUCTS OF GLUTEN FROM TOXIC WHEAT

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(Received for publication July 28, 1936)

It is quite well established that the selenium present in plants grown on soils containing this element, particularly in the seeds of such plants, is chemically tied up with protein. When gluten from toxic wheat is completely hydrolyzed and the hydrolysate subjected to analyses according to methods generally used in the separation of amino acids from proteins, it is found that the selenium-containing compound is largely concentrated in the leucine fraction, which usually also contains some valine, phenylalanine, and traces of other amino acids. Two difficulties are early confronted when attempting to isolate and characterize this compound. In the first place, the quantity of selenium present is relatively small. Again, the properties of the selenium compound are apparently very similar to those of the amino acids associated with it. Any procedure which will yield a fraction having a relatively high concentration of selenium should facilitate the isolation and identification of the compound.

Jones and Gersdorff (1934) showed that a mild peptic digestion of casein yields a partially digested product which is free from a detectable quantity of cystine. Because of the similarity of sulfur and selenium with respect to their general properties, it was conceived that by a similar procedure of partial digestion applied to the gluten from seleniferous wheat a fraction of the protein might be obtained in which most of the selenium would be concentrated. Furthermore, a study of the relative distribution of cystine and selenium in the separable fractions of the partially digested gluten might reveal a correlation which would

throw light on a possible relation between the selenium and sulfur in the protein molecule.

The results obtained in this investigation show that by digesting gluten from seleniferous wheat with pepsin for a short period partially hydrolyzed protein fractions are formed which can be separated. Two of the four fractions separated were free from selenium, one free and the other almost free from cystine. Most of the selenium in the gluten used was concentrated in one small fraction. By removal of the dicarboxylic amino acids from this fraction a further concentration of selenium was accomplished.

These results also contribute additional evidence that the selenium in toxic wheat is an integral part of the protein.

Preparation of the Gluten

The gluten used was prepared from toxic wheat supplied by K. W. Franke of the South Dakota Agricultural Experiment Station. The wheat was of the same lot as that employed previously in this laboratory by Horn, Nelson, and Jones (1936). The starch was washed out as completely as possible in the usual manner from 2 kilos of flour [1] prepared from this wheat. One hundred and ninety-nine grams of dry gluten were obtained.

In order to dehydrate the moist gluten obtained after washing out the starch, it was cut into small particles and allowed to stand overnight in about 6 liters of absolute alcohol to which sufficient lithium chloride had been added to cause a separation of the small amount of gliadin which had dispersed because of dilution of the alcohol by the water in the moist gluten. The treatment of the gluten with absolute alcohol was repeated several times using successively smaller volumes of alcohol until the gluten was thoroughly dehydrated. An additional amount of lithium chloride was added to the first alcoholic washing, and the small quantity of material which separated was added to the main portion of the gluten. The dehydrated gluten, moist with alcohol, was finely ground in a hand mill, and the absorbed alcohol was removed by washing the product with absolute ether. The gluten was finally dried in an oven at 110° C., and then exposed to the air for 36 hours in order to come to moisture equilibrium so as to permit accurate weighing of the samples.

Digestion with Pepsin

Eighty grams of the air-dry gluten, moistened with a little alcohol, was heated at 80° C. in 1,900 cc. of 1/10 N hydrochloric acid until all the gluten was dispersed. This required about 2½ hours. The

[1] We are indebted to the Bureau of Agricultural Economics for preparation of the flour in their Milling, Baking, and Chemical Laboratory.

mixture was then cooled to 38° C. and 2 g. of pepsin in 100 cc. of 1/10 N hydrochloric acid at the same temperature was added, and the mixture was digested for 3 hours at 37 to 38° C. It was then heated to 80° C. for a few minutes in order to inactivate the enzyme. The final volume was 2,144 cc. Two aliquots of 26.8 cc., each representing 1 g. of the air-dry gluten, were removed for some preliminary determinations. The amount remaining in the digest represented 78 g. of the gluten upon which all subsequent calculations were based.

FRACTION A. At the end of the digestion period a quantity of flocculent material had separated. This was removed by centrifugation and washed five times with distilled water. The air-dry material weighed 2.5 g.

FRACTION B. The filtrate and washings from Fraction A were brought to pH 6.2 with 5 normal sodium hydroxide and 20% hydrochloric acid. It had been previously ascertained that the maximum amount of this material was precipitated at pH 6.2. The precipitate was removed by centrifugation and washed successively with five portions of dilute acetic acid at pH 6.2. The material, dried in the usual way with alcohol and ether, weighed 9.0 g.

FRACTION C. The filtrate plus the washings from Fraction B were concentrated by rapid evaporation to about one-half of the original volume. On standing at room temperature (23°) a flocculent material separated, which was separated, washed, and dried. The yield was 1.0 g.

FRACTION D. The soluble material remaining in the filtrate and washings from Fraction C is referred to as Fraction D. The solution was concentrated by rapid evaporation, and the volume was made up to 500 cc. Aliquots of this solution were used for analyses.

The four fractions were first tested qualitatively for the presence of selenium by the codeine sulfate test as developed by Horn (1934). This test is very reliable for establishing the absence of selenium, as the presence of a trace of this element will give a characteristic blue color with codeine sulfate under the conditions of the test. Entirely negative results were obtained with Fractions A and C, but Fractions B and D gave strong positive tests. Selenium was then determined quantitatively in Fractions B and D by a method involving titration with standard sodium thiosulfate. Details of this method will be published later.

Cystine was determined by the Sullivan (1929) method.

The yields of the fractions and the results of their analyses are summarized in Table I.

There are two features of particular interest in connection with the results obtained in this study. Fifty-eight % of the total selenium in

the gluten used was concentrated in Fraction B, which weighed 9 g. and which represents only 11.5% of the gluten. This concentration of selenium in Fraction B corresponds to 1,000 parts per million. The other feature deserving emphasis is that the fractions which contained no selenium were also lacking in cystine. No trace of selenium or cystine was found in Fraction A. Fraction C was also devoid of selenium, and contained only a trace of cystine.

TABLE I

PEPTIC DIGESTION OF GLUTEN (78 G.) FROM SELENIFEROUS WHEAT

Fraction	A	B	C	D
Weight of fractions, g.	2.50	9.00	1.00	65.5
Fractions as % of gluten used	3.20	11.54	1.28	83.98
Selenium, *mg.*	0.00	9.00	0.00	6.40
Percent in the fractions of total selenium in gluten used	0.00	58.40	0.00	41.60
Cystine, *mg.*	0.00	46.30	4.22	394.56
Percent in the fractions of total cystine in gluten used	0.00	10.50	0.95	88.64
Cystine, %	0.00	0.51	0.42	0.61

The dicarboxylic amino acids can be separated as a group from the other products of hydrolysis of a protein by precipitation of their barium salts in aqueous solution by addition of alcohol. It has been repeatedly found that the dicarboxylic amino acid fraction can be removed in this way from the completely hydrolyzed toxic gluten entirely free from selenium.

It was accordingly of interest to determine the amount of dicarboxylic amino acids in Fraction B, because removal of this group of amino acids would readily afford a means of obtaining a fraction from the toxic gluten having a still higher concentration of selenium. Five grams of Fraction B was hydrolyzed by boiling with 40% sulfuric acid for 36 hours. The sulfuric acid was then quantitatively removed with barium hydroxide and the dicarboxylic amino acids were removed in the usual way. They weighed 0.754 g. and were found to be practically free from selenium. The original 9 g. of Fraction B, therefore, contained 1.36 g. of dicarboxylic amino acids, equivalent to 15%, leaving the residual part of Fraction B with a selenium content of 1,178 parts per million.

Summary

Gluten prepared from toxic wheat grown on seleniferous soil was digested for 3 hours by pepsin. Four fractions of partial hydrolysis products of the protein were separated. Fraction A (2.5 g.) was free from both selenium and cystine. Fraction C (1 g.) was also devoid

of selenium and contained only a trace of cystine. Fraction B (9 g.)' representing in weight 11.5% of the gluten used, contained 58% of the total selenium and 10.5% of the total cystine.

By removing the dicarboxylic amino acids from Fraction B, the residual part of the fraction contained 1,178 parts per million selenium.

The results show, (*a*) that over half of the selenium in the toxic gluten can be concentrated in a small partial hydrolysis fraction, (*b*) an interesting relation in the distribution of selenium and cystine in the hydrolysis products. They also contribute further evidence that the selenium in toxic wheat is combined with the protein.

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